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Kinetics of Transmission, Infectivity, and Genome Stability of Two Novel Mouse Norovirus Isolates in Breeding Mice

Jennifer A Kelmenson,[1] Darcy P Pomerleau,[1] Stephen Griffey,[3] Weidong Zhang,[2] Michele J Karolak,[4] and James R Fahey[1,*]

Murine noroviruses are a recently discovered group of viruses found within mouse research colonies in many animal facilities worldwide. In this study, we used 2 novel mouse norovirus (MNV) wildtype isolates to examine the kinetics of transmission and tissue distribution in breeding units of NOD.CB17-*Prkdc^{scid}*/J and backcrossed NOD.CB17-*Prkdc^{scid}*/J × NOD/ShiLtJ (N1) mice. Viral shedding in feces and dissemination to tissues of infected offspring mice were monitored by RT-PCR over a 6-wk period postpartum. Histologic sections of tissues from mice exposed to MNV were examined for lesions and their sera monitored for the presence of antibodies to MNV. Viruses shed in feces of parental and offspring mice were compared for sequence homology of the *Orf2* gene. Studies showed that the wildtype viruses MNV5 and MNV6 behaved differently in terms of the kinetics of transmission and distribution to tissues of offspring mice. For MNV5, virus transmission from parents to offspring was not seen before 3 wk after birth, and neither isolate was transmitted between cages of infected and control mice. Susceptibility to infection was statistically different between the 2 mouse strains used in the study. Both immunodeficient NOD.CB17-*Prkdc^{scid}*/J mice and NOD.CB17-*Prkdc^{scid}*/J × NOD/ShiLtJ offspring capable of mounting an immune response shed virus in their feces throughout the 6-wk study period, but no gross or histologic lesions were present in infected tissues. Progeny viruses isolated from the feces of infected offspring showed numerous mutations in the *Orf2* gene for MNV5 but not MNV6. These results confirm previous studies demonstrating that the biology of MNV in mice varies substantially with each virus isolate and mouse strain infected.

Abbreviations: MNV, murine norovirus; MLN, mesenteric lymph nodes; NOD-scid, NOD.CB17-*Prkdc*^{scid}/J; VP1, viral protein 1.

The recent discovery of murine-specific noroviruses[15] has stimulated concern in the laboratory animal health community regarding the potential for this group of viruses to cause disease in breeding colonies of mice or to negatively impact research with mice from norovirus infected colonies. Current knowledge of the biology of noroviruses in mice (MNV) is constrained by the limited number of virus isolates and mouse strains studied. One study[15]described the biologic and physicochemical properties of the original MNV1 isolated from mice deficient in a specific innate immune function. More recently, this innate immune deficiency has been mapped to STAT1 regulation of IFNαβ secretion.[21]

Previous work[15] demonstrated that inoculation of MNV1 into mouse strains deficient in the acquired immune response (129 RAG $2^{-/-}$, B6 RAG$1^{-/-}$) resulted in the development of persistent infections with no evidence of disease, whereas inoculation of fully immunocompetent mice (129S6/SvEvTac) resulted in rapid elimination of MNV1, with viral RNA undetectable in the viscera by 3 d after inoculation. More recently, infections of outbred immunocompetent mouse strains with 3 wildtype isolates of MNV obtained from different geographic areas of the United States have been described.[11] Virus was detected in the feces and tissue of infected mice throughout the 8-wk study, suggesting that some isolates of MNV may persistently infect immunocompetent mice.

The purpose of the present investigation was to extend the current knowledge of MNV by using 2 isolates of the virus in mouse strains that have not been previously used as infection models for MNV. We examined natural virus transmission from infected breeders to offspring, kinetics of infection within litters of infected breeding mice, and the pathogenesis of infection in breeding colonies of mice. In addition, we examined the effect of virus passage from parents to offspring on genomic stability of these 2 viral isolates. Exposure of offspring of immunodeficient mice and immunocompetent mice to the 2 different isolates of MNV resulted in different patterns of virus transmission, susceptibility to infection and kinetics of infection as shown by the progressive spread of virus within litters and in intestinal and extraintestinal tissues. MNV was shed persistently in the feces of all mice tested regardless of immune status, and viral progeny isolated from offspring mice contained genome sequence differences from the parent virus in the *Orf2* gene, an area of the MNV genome known to be susceptible to mutations.

Materials and Methods

Isolation of novel murine norovirus genotypes. Live mice and frozen mouse tissues (spleen, intestine, and feces) from several strains of mice were provided to the Diagnostic Laboratory at The Jackson Laboratory (Bar Harbor, ME) by Ms Lynne Bauer (John G Rangos Sr Research Center, Children's Hospital of Pittsburgh,

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[1]Laboratory Animal Health Services Department and [2]Computational Services, The Jackson Laboratory, Bar Harbor, Maine; [3]Comparative Pathology Laboratory, University of California, Davis, California; [4]Maine Medical Research Institute, Scarborough, Maine.
**Corresponding author. Email: jim.fahey@jax.org*

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PA). Prior serologic testing of mice from some of these colonies had demonstrated antiMNV antibodies (Research Animal Diagnostic Laboratory, Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri, Columbia, MO). RNA was extracted from tissues of MNV-seropositive and -seronegative mice from the John G Rangos Sr Research Center by using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA was used in RT-PCR for detection of MNV, as described in the next section.

RT-PCR and nucleotide sequencing. RT-PCR reactions were carried out by using a OneStep RT-PCR Kit (Qiagen), according to the manufacturer's recommendations, with a 1× final concentration of Q solution and 0.6 µM of each primer. Initial primer pairs for RT-PCR were selected from published MNV1 data[15] (GenBank accession number, NC008311) by using Primer Express Software version 3.0 (Applied Biosystems, Foster City, CA) to amplify and sequence portions of *Orf*1 and *Orf*2 from MNV5 and MNV6. RT-PCR products were purified by using ExoSAP-IT (USB, Cleveland, OH) or by cloning by using a TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA), followed by transformation and plasmid purification by using a Plasmid Mini Kit (Qiagen). Cycle sequencing reactions were performed by using a BigDye Terminator Chemistry v3.1 Cycle Sequencing Kit (Applied Biosystems). Excess dye terminators were removed by using CleanSEQ (Agincourt Bioscience, Beverly, MA). Purified sequencing reactions were loaded and run on an automated sequencer (3730xl DNA Analyzer, Applied Biosystems). The resulting raw data were analyzed by using Sequencing Analysis Software version 5.2 (Applied Biosystems). All nucleotide and amino acid sequence data alignments were accomplished by using Sequencher 4.1.4 (Gene Codes, Ann Arbor, MI). Each sequence was confirmed by using multiple amplification products, which, when aligned, generated overlapping nucleotide data for all sequenced sections, to exclude changes due to lack of fidelity of the reverse transcriptase.

Sequence comparisons among MNV1, MNV5, and MNV6 showed high levels of nucleotide sequence similarity (greater than 90% in *Orf1* and *Orf*2) and allowed for the design of multiple primer pairs in conserved regions of these MNV genomes. Two primer pairs, set 1 (forward, 5′ TTG TTC TGG AGG CCG AAA TCA T 3′; reverse, 5′ AAT TTC ATC TTG GCA CCG CA 3′) and set 2 (forward, 5′ GCC TCC TTT ATC CTT CTG ACA CAG TT 3′; reverse, 5′ CGC CGC ATA GAT TTC CTG GT 3′), were used for subsequent detection of MNV5 and MNV6, respectively, in experimentally inoculated animals (see next section). Different primer pairs (data not shown) were used for comparison of the *Orf*2 gene sequences in viral progeny from specimens of parental and offspring of both mouse strains. The capsid gene of MNV (*Orf*2) from start codon to stop codon was used as the target region for sequencing, because this gene is often used as the reference region for genotyping human norovirus isolates[1,14,16,25,30] and is known to be a region with potentially high sequence variability.[29]

The PCR thermal cycling conditions were: reverse transcription for 30 min at 50 °C; DNA polymerase activation and initial denaturation for 15 min at 95 °C; 40 cycles of 30 s at 94 °C (DNA melting), 30 s at 55 °C (primer annealing), and 1 min at 72 °C (strand extension); and final extension of 10 min at 72 °C. Products were separated on a 2% SeaKem LE (Cambrex Bio Science, Rockland, ME) agarose gel containing ethidium bromide and visualized under UV light.

Viral cell culture. Novel MNV isolates were cultured in RAW 264.7 cells obtained from the American Type Culture Collection (catalog TIB71, Manassas, VA). Cells were incubated in DMEM (American Type Culture Collection) containing 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate and supplemented with 10% FBS (Invitrogen). Cells were grown to confluency in T25 flasks (Corning, Corning, NY) and the media replaced every 2 to 3 d. To determine the viability of viruses shed in feces from recently infected pups, fecal pellets from infected and control animals were ground by using a rotostator homogenizer with Omni-tips Disposable Rotar Stator Generator Probes (Omni International, Warrenton, VA) in 1 ml of sterile PBS. Fecal debris was pelleted by centrifugation at 14,000 × *g* for 3 min, and the resulting supernatant was removed, applied to a 0.45-µm centrifuge-tube filter (Costar SpinX, Corning) and spun at 14,000 × *g* for 1 min. The filtrate then was applied to a 0.22-µm centrifuge tube filter (Costar SpinX, Corning) and spun at 14,000 × *g* for 1 min; 50 µL of this sterile-filtered viral suspension was diluted in 1 mL DMEM (without FBS) for inoculation of a single T25 flask of RAW 264.7 cells. Filtrates from fecal samples were applied to monolayers of RAW 264.7 cells after gently washing with unsupplemented DMEM. The diluted filtrates were added to flasks and incubated for 1 h at 37° for viral adsorption. After adsorption, the virus suspension was removed, the flasks washed with DMEM, and 10 mL of supplemented DMEM was added back to the flask. The cells were monitored daily for evidence of cytopathic effects.

Mice. All mice used in this study were obtained from production colonies at The Jackson Laboratory. The mice were free of virus antibody, bacterial pathogens, and parasites as determined by routine health monitoring of the production colonies. Two strains of mice, NOD.CB17-*Prkdc*^{scid}/J (NOD-scid), a strain that is immunodeficient due to a naturally occurring mutation to the protein kinase, DNA activated, catalytic polypeptide gene (homozygous mice are deficient in mature B and T cell function), and the NOD/ShiLtJ strain, the primary genetic background strain for NOD-scid mice, were used throughout these studies. Mice were housed in AAALAC-approved animal facilities using animal care programs in compliance with the Public Health Service *Guide for the Care and Use of Laboratory Animals*.[22] This study was reviewed and approved by the institutional animal care and use committee. Mice were housed in plastic duplex mouse boxes (5 in. × 12 1/8 in. × 6 in.) covered with wirebar lids; half of the control cages were covered with filter tops also. Mouse boxes were kept within flexible-film isolators (Harlan Isotec System, Bicester, England) with feed and acidified water (pH range, 2.70 to 3.15). Mouse boxes, feed, and bedding were delivered within a transport isolator that docked with the flexible-film isolator port. Mice were placed in glass jars, which were dipped in a general-purpose disinfectant (Wescodyne, Steris, Mentor, OH) and then passed into the flexible-film isolators. Mouse boxes (including bedding, water, and chow) were changed biweekly. Mice received feed and water ad libitum.

Serologic testing. All immunocompetent mice to be used as breeders were prescreened serologically and were negative for the presence of antiMNV antibodies prior to the start of experiments. Blood samples were obtained from mice by means of the retroorbital plexus after treatment with topical tetracaine (Tetracaine Ophthalmic Solution, Bausch and Lomb, Tampa, FL) in the eyes. Blood samples were allowed to clot at room temperature and the sera collected after centrifugation. The serum samples were

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tested for antibodies to MNV at the Research Animal Diagnostic Laboratory (Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri, Columbia, MO). Serologic screening of male breeding mice (groups 2 and 6) and randomly selected immunocompetent offspring of MNV-infected breeders also was performed during the course of experimentation. Immunodeficient mice used as breeders were prescreened for MNV by RT-PCR of fresh fecal samples.

Experimental design. Colonies of infected NOD-scid mice were established in 2 ways: by feeding tissues from neonatal mice of the same mouse strain that had been inoculated intracerebrally with MNV 5 and by gavage of female breeding mice with fecal and intestinal homogenates from the original tissues from which the viruses were isolated (see next section). In both cases, the original virus isolates were used without passage in cell culture. The virus-exposed female mice were tested by weekly fecal RT-PCR for virus shedding. MNV-exposed female mice showing positive fecal RT-PCR results were used as breeders for virus transmission experiments. Eight groups of mice were used to study virus transmission from parents to offspring within a breeding colony. A description of the MNV5- and MNV6-exposed mice and their controls is presented in Table 1. Within each isolator, control mice were housed on shelves below the infected breeding mice, with half of the control cages covered with filter tops over the wire-bar lid so that we could determine whether MNV was transmitted through environmental contamination of cages. During cage changes and sampling of animals, controls were handled first by using the gloves attached to the isolators. Gloves were disinfected between cages by using 70% ethanol. Mice were housed with both male and female breeders present with the offspring.

Breeding mice were monitored for virus shedding on a weekly basis by fecal RT-PCR. Pups born to virus-exposed and control breeders were tested on a weekly basis for the presence of virus in 2 ways: fresh fecal samples were taken from every pup in each litter for RT-PCR testing, and pups were selected randomly from each litter each week and euthanized. The number of pups sampled per time point is described in the legends to figures and tables. Samples of tissues and feces were removed from the euthanized pups at necropsy for viral RT-PCR, and tissues also were used for histopathology.

In most cases throughout these experiments, breeding was allowed to occur continuously, to mimic the breeding scenario in standard mouse rooms. Offspring were weaned at 4 wk of age, segregated according to sex, and kept as littermates (no pooling of litters). Female mice were bred postpartum, with only occasional overlaps of 1 to 2 d between new and older litters within the same cage. Breeders were separated or euthanized when examined for viral infection and when it was necessary to reduce the mouse population within an isolator.

Histologic examination. Liver, spleen, mesenteric lymph nodes (MLN), and the entire intestinal tract (dissected into duodenum, jejunum, ileum, cecum, and colon) were taken from experimental mice, fixed in Telly's fixative (100 mL 70% ethanol, 5 mL formalin, and 5 mL glacial acetic acid), and embedded in paraffin for sectioning and staining. All sections were stained with hematoxylin and eosin and examined in a blinded fashion by a veterinary pathologist.

Statistical testing. All statistical evaluations in this study were performed with JMP version 6.0.3., an interactive statistical and graphics package (SAS, Cary, NC). The Log-Likelihood Ratio Chi Square test was used to determine whether there was an association between susceptibility to infection with either MNV isolate used in this study and the mouse strains exposed to the viruses. We used a complete model that included strain, age, and strain×age interaction effects. A backward elimination strategy was used to drop one insignificant term at a time.[34] The Log-Likelihood Ratio Chi Square test was used to determine whether there was an association between the distribution of each virus isolate to MLN and the infected mouse strain.

Table 1. Description of experimental groups exposed to MNV5 or MNV6 and controls

Group	Mice
1	♀ NOD-scid infected (MNV5) × ♂ NOD-scid uninfected
2	♀ NOD-scid infected (MNV5) × ♂ NOD/ShiLtJ uninfected
3	♀ NOD-scid × ♂ NOD-scid uninfected controls
4	♀ NOD-scid × ♂ NOD/ShiLtJ uninfected controls
5	♀ NOD-scid infected (MNV6) × ♂ NOD-scid uninfected
6	♀ NOD-scid infected (MNV6) × ♂ NOD/ShiLtJ uninfected
7	♀ NOD-scid x ♂ NOD-scid uninfected controls
8	♀ NOD-scid x ♂ NOD/ShiLtJ uninfected controls

RESULTS

Isolation of novel MNV genotypes. Mouse tissues and mice from research colonies at the John G Rangos Sr Research Center (Children's Hospital of Pittsburgh, PA) were used as a source from which to isolate viruses. Intestinal tissue and feces were screened for murine noroviruses by RT-PCR using several primer pairs designed from the MNV1 genome. Two novel isolates of MNV (MNV5: GenBank accession number, EF650480; and MNV6: EF650481) were detected in intestinal samples from 2 different mouse strains: MNV5 from the BetaSarca-Glycan knockout-5 strain of mice and MNV6 from GDF-8R knockout-1155 N2 mice. Neither of these mouse strains is known to be deficient in innate immune functions.[3]

Transmission of novel MNV isolates in breeding mouse colonies. To determine in vivo biologic characteristics of the 2 novel MNV isolates, including kinetics of transmission in breeding mice, we used the immunodeficient mutant mouse strain NOD-scid and N1 mice from a backcross of NOD-scid and its principle genetic background strain NOD/ShiLtJ as a control. Figure 1 A shows the kinetics of infection among litters of mice born to MNV5-infected breeders as determined by randomly sampled fresh feces from offspring at 3 to 6 wk postpartum. Figure 1 B shows infection kinetics data for tissues taken from offspring of MNV5-infected dams during this same period. The natural transmission of MNV5 from infected dams to their male partners and to offspring readily occurred. Male partners of breeding dams were infected within 1 wk (data not shown), independent of the strain used. The number of infected offspring (8 litters tested for Group 1 and 6 litters for Group 2) progressively increased over the testing interval, with 100% of the Group 2 pups infected by week 5 and 80% of the Group 1 pups infected by week 6 (Figure 1 A, B). In both fecal and tissue sampling, the rate of infection was consistently more rapid ($P < 0.05$) among Group 2 pups compared with group 1 pups. Twelve mice were tested for MNV5 between birth and 14 d of age, and all were negative by both fecal and tissue RT-PCR.

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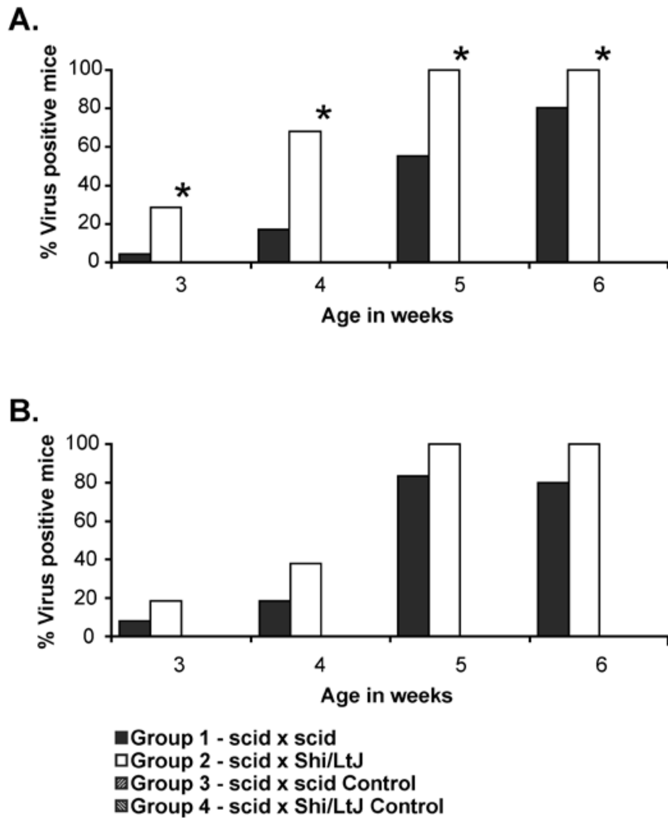

Figure 1. Percentage of pups positive by (A) fecal or (B) tissue RT-PCR for the presence of MNV5. Fecal RT-PCR testing was done on samples from all pups born to infected or control uninfected mice. Tissue RT-PCR was done on randomly sampled pups from each litter. The number of pups tested for fecal virus at each time point (week 3 through 6) ranged from 14 to 46. For uninfected controls, the number of pups sampled ranged from 7 to 24. For tissue RT-PCR, the number of samples per time point ranged from 7 to 13. The tissues tested for MNV5 included liver, spleen, MLNs, duodenum, jejunum, ileum, cecum, and colon. Any single RT-PCR-positive tissue was considered a positive finding for the animal. *, significant difference for fecal RT-PCR: week 3, $P = 0.03$; week 4, $P < 0.0001$; week 5, $P < 0.0001$. No statistically significant differences were seen in the tissue RT-PCR.

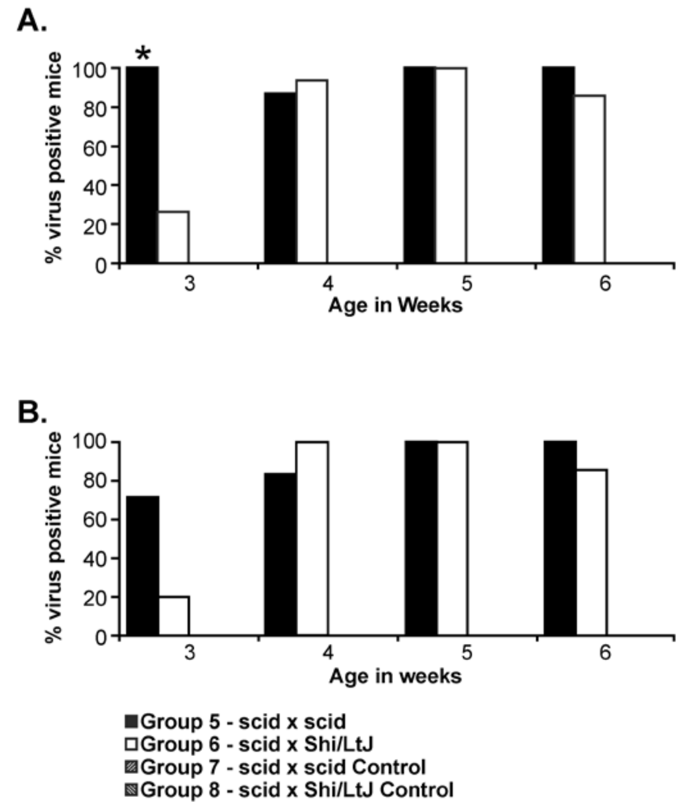

Figure 2. Percentage of pups positive by (A) fecal or (B) tissue RT-PCR for the presence of MNV6. Fecal RT-PCR testing was done with samples from all pups born to infected or control uninfected mice. Tissue RT-PCR was done on randomly sampled pups from each litter. The number of pups test for fecal virus at each time point (week 3 through 6) ranged from 7 to 15. For uninfected controls, the number of pups sampled ranged from 6 to 20. For tissue RT-PCR, the number of samples per time point ranged from 4 to 12. The tissues tested for MNV6 included liver, spleen, MLNs, duodenum, jejunum, ileum, cecum, and colon. Any single RT-PCR-positive tissue was considered a positive finding for the animal. *, significant difference for fecal RT-PCR (week 3, $P < 0.0002$). No statistically significant differences were seen in the tissue RT-PCR.

For this reason, sampling was not continued during weeks 1 and 2 postpartum.

The calculated likelihood ratio χ^2 test scores for the fecal infection data showed that group 2 mice (NOD-scid × NOD/ShiLtJ) were significantly more susceptible ($P < 0.0001$) to infection with MNV5 than were mice in group 1 (NOD-scid × NOD-scid). Similar evaluation of tissue RT-PCR data again showed that compared with group 1 mice, group 2 mice were more susceptible ($P = 0.0430$) to MNV5. Susceptibility to infection also showed an age effect according to results from fecal RT-PCR, in that significant differences between mouse strains could be seen beyond 3 wk of age (Figure 1). However, this difference is not significant according to tissue RT-PCR data, perhaps because fewer mice were tested per time point than for fecal virus.

Figure 2 demonstrates the kinetics of norovirus infection among offspring of dams infected with MNV6. The natural transmission of MNV6 from infected dams to their male partners and offspring readily occurred. The kinetics of infection among offspring (5 litters each tested for groups 5 and 6) were more rapid ($P < 0.05$) than those with MNV5 in that 100% of the group 5 (NOD scid × NOD scid) offspring sampled for fecal virus shedding (Figure 2 A) were positive at week 3 and remained at a high level of infection thereafter. Among the group 6 offspring (NOD-scid × NOD/ShiLtJ), transmission to more than 90% of the sampled offspring took slightly longer, with 94% infected at 4 wk of age. Between birth and 15 d of age, only 1 of the 2 mice that were tested at day 14 was found to be infected (see discussion for details). No additional mice were tested for MNV6 during the first 2 wk postpartum. Group 5 mice were significantly ($P < 0.008$) more susceptible to MNV6 infection than were group 6 mice, and age exerted an effect on MNV6 infection, but only at 3 wk. The corresponding differences in the tissue RT-PCR data were not statistically significant, likely because fewer mice were tested per time point than were for fecal RT-PCR. At no time during these experiments were control mice positive for MNV by RT-PCR of fecal or tissue samples, regardless of whether (or not) they were housed in filter-covered cages.

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Table 2. Tissues of group 1 pups that were positive for MNV5 RNA by RT-PCR

Day	Duodenum	Jejunum	Ileum	Cecum	Colon	MLN	Liver	Spleen
17			X	X	X	X		
24			X	X	X			
26			X	X	X			
29		X	X	X	X			
29						X		
30	X	X	X	X	X	X		
32	X		X	X	X			
32				X	X			
32				X	X			
33	X	X	X	X	X	X		
33			X	X	X			
36	X	X	X	X	X			
36	X	X	X	X	X			X
36	X		X	X	X	X	X	
36	X		X	X	X	X		
36	X		X	X	X	X		
37				X	X			
37	X	X		X	X			
41	X	X	X	X	X	X		
41	X	X	X	X	X			
42	X		X	X	X	X		X
42	X		X	X	X			
42	X		X	X	X			
43	X	X	X	X	X			
43	X		X	X	X			
44	X		X	X	X			
48				X	X	X		

On each day, total RNA was isolated from the designated tissue and probed by specific primers for the presence of MNV5.

Previous studies of MNV in mice[11,15,31,33] have shown variable patterns of virus dissemination in the tissues of infected mice, depending on the strain of mouse used and the particular virus isolate. Tables 2 and 3 demonstrate the tissue distribution of MNV5, as determined by RT-PCR, among offspring in groups 1 and 2, respectively. In both experimental groups, viral RNA was found in all segments of intestinal tissues and was present most consistently in the ileum, cecum, and colon starting at 3 wk of age and extending to the end of the study. However, approximately twice the number of sampled mice in group 2 (16 of 23, 69.6%) had RT-PCR evidence of MNV in the MLN from 3 to 6 wk after exposure to infected dams, compared with group 1 mice (10 of 27, 37.0%; $P < 0.0001$). In addition, sporadic samples of liver and spleen from mice in both groups 1 and 2 were positive for viral RNA.

The tissue distribution of viral RNA in offspring mice exposed to MNV6-infected dams is shown in Tables 4 and 5. As with MNV5-exposed mice, among RT-PCR positive mice, MNV6 RNA was found most consistently in the ileum, cecum, and colon, from the initial sampling to the end of the study. In addition, the duodenum, jejunum, and MLN frequently were positive for the presence of viral RNA from 3 to 6 wk postnatally. The percentage of offspring with virus disseminated to the MLN was 64.3% for group 5 mice and 90% for group 6 mice ($P < 0.0001$).

Detection of live virus by cell culture. Sterile filtered homogenates from fecal samples randomly collected from pups exposed to either MNV5- or MNV6-infected dams were cultured on RAW 264.7 cells for determination of fecal virus viability. In all cases, the fecal pellets collected at weeks 3 to 6 postnatally were positive for live virus from both MNV5- and MNV6-infected pups (data not shown). Control offspring that were exposed to uninfected dams were free of live virus.

Histopathology. At intervals during the study, pups from each experimental group were euthanized and tissues collected for RT-PCR and histopathology. Examination of histologic slides in a blinded fashion demonstrated the absence of virus-specific lesions in the intestine, liver, spleen, and MLN of infected mice irrespective of immune status (data not shown).

Serologic response. Several immunocompetent breeding animals and offspring randomly selected from experimental groups were bled and their serum tested for antibodies to MNV. In this study, the antigen used in the detection method was MNV1. Serologic responses to infection with either MNV5 (group 2) or MNV6 (group 6) by multiplex fluorescent immunoassay did not become evident until approximately 6 wk of age (Table 6). This result is consistent with a 3-wk postinfection antibody response. All immunocompetent male breeders were tested immediately prior to their removal from the study (that is, 119 to 189 d after exposure to infected dams). In all cases, these male mice were antibody-positive to the MNV1 antigen.

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Table 3. Tissues of group 2 pups that were positive for MNV5 RNA by RT-PCR

Day	Duodenum	Jejunum	Ileum	Cecum	Colon	MLN	Liver	Spleen
19			X	X	X			
21	X			X	X	X	X	
24				X				
24	X	X	X	X	X	X		
24	X	X	X	X	X	X		
34	X		X	X	X	X		
34		X	X	X	X	X	X	
34		X	X	X	X	X		
34		X	X	X	X			
34		X	X	X	X	X	X	
34		X	X	X	X	X		
34		X	X	X	X	X	X	
37	X	X	X	X	X	X		
37	X	X	X	X	X	X		
38				X	X	X		
39		X	X	X	X	X		
39	X	X	X	X	X			
41	X	X	X	X	X			
41	X	X	X	X	X	X		
46	X		X	X	X			
46	X	X	X	X	X	X		
47	X	X	X	X	X			
47		X	X	X	X	X		

On each day, total RNA was isolated from the designated tissue and probed by specific primers for the presence of MNV5.

Comparison of viral nucleic and amino acid sequences. The *Orf2* and partial *Orf1* genes of samples of MNV5 and MNV6 were sequenced and their nucleotide sequences registered in GenBank (accession numbers EF650480 and EF650481, respectively). Both MNV isolates had greater than 90% sequence homology with MNV1, the prototype strain. Progeny virus shed from MNV5- and MNV6-infected breeding mice and infected offspring were partially sequenced to determine whether mutations were evident after a single round of virus infection of parental mice and subsequent transmission and replication in offspring. To this end, nucleotide sequence data from viruses in fecal samples taken from breeders were pooled and analyzed, as were nucleotide sequence data from viruses in fecal samples from offspring. Table 7 shows those nucleotide positions in the *Orf2* sequences of MNV5 and MNV6 that differed between viruses shed by breeding mice and their offspring. The *Orf2* nucleotide sequence of MNV5 viruses isolated from feces of offspring showed more single-nucleotide mutations (that is, 21 substitutions) than did the same region in MNV6 (5 substitutions). The predicted amino acid sequences revealed that 5 of the 21 mutations in MNV5 lead to amino acid changes, compared with 2 of the 5 MNV6 mutations.

Discussion

Murine noroviruses are highly prevalent within rodent housing facilities of many research institutions[11]. The discovery of new isolates will eventually allow mapping of the molecular epidemiology of MNV isolates found in North America and elsewhere. Epidemiologic characterization likely will include information on transmission and pathogenicity, if any, of new virus isolates in different mouse strains. Characterization also should include genomic sequence data. In this regard, this report describes the first study undertaken to characterize 2 novel isolates of MNV in breeding mice and includes information regarding infectivity, persistence of infection, and tissue distribution in 2 mouse strains and changes in the viral genome after passage from parents to offspring.

Transmission of both the MNV5 and MNV6 wildtype isolates used in this study readily occurred from infected female breeders to cohoused uninfected male breeders and the offspring of these matings. However, viral transmission was not detected in cages of control animals, even when filter tops were not provided. This finding suggests that appropriate husbandry practices are sufficient to prevent cage-to-cage transmission of MNV. Among mice infected with MNV5, offspring were not positive for fecal or tissue virus as determined by RT-PCR until approximately 3 wk postpartum, suggesting that the offspring become infected via coprophagy. Therefore, ingesting virus-contaminated feces from infected parents may be a primary means of spreading the virus within a cage of mice. The presence of maternal antibodies in pups from both mouse strain crosses used in this study can probably be ruled out as a cause of the delay in MNV5 transmission from birth to approximately 3 wk of age, because the female breeders in all cases were NOD-scid mice: mice of this strain, which are severely immunodeficient, are incapable of developing antibodies. Compared with scid mice on other strain backgrounds, NOD-scid mice have a very low rate of reversion to production of mature lymphocytes.[12] Additional studies in which sera from infected NOD-scid female breeders are evaluated for

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Table 4. Tissues of group 5 pups that were positive for MNV6 RNA by RT-PCR

Day	Duodenum	Jejunum	Ileum	Cecum	Colon	MLN	Liver	Spleen
17			X	X				
17			X	X				
21	X		X	X	X	X		
21	X		X	X	X	X		
21				X	X			
22	X	X	X	X	X			
22	X	X	X	X				
24			X	X	X	X		
24			X	X	X	X		
24				X				
32	X	X	X	X	X			
33	X	X	X	X	X	X		X
33	X	X	X	X	X	X	X	X
35				X	X	X		X
35	X	X	X	X	X			
35	X	X	X	X	X	X		
36	X	X	X	X	X	X	X	
36	X	X	X	X	X	X	X	
36	X	X	X	X	X	X		
36	X	X	X	X	X	X	X	
36	X	X	X	X	X	X	X	X
37	X	X	X	X	X	X		
37	X	X	X	X	X	X	X	
37	X	X	X	X	X			
37	X	X	X	X	X	X		
39	X	X	X	X	X			
39	X		X	X	X	X		
41	X	X	X	X	X	X		

On each day, total RNA was isolated from the designated tissue and probed by specific primers for the presence of MNV6.

antibodies to MNV would enable us to rule out that antibodies were involved in this aspect of virus transmission. With regard to MNV6, additional studies will be required to determine whether it transmits to mice between the ages of birth to 3 wk.

In vivo characterization of MNV5 and MNV6 in mice showed that the MNV5 isolate transmitted in a slow progressive fashion among the litters of groups 1 and 2, whereas MNV6 transmission progressed very rapidly within litters (in groups 5 and 6). Interestingly, the group 2 backcross mice (NOD-scid × NOD/ShiLtJ) exposed to MNV5 had a consistently greater percentage of infected pups at each time point than did group 1 pups (NOD-scid × NOD-scid), as confirmed by both fecal and tissue viral RNA analysis. Perhaps parental mice shed higher numbers of virus-laden feces, or more viruses per fecal pellet, in group 2 versus group 1 mice, leading to the exposure of more pups to infectious virus or to more infectious virus particles per exposure. Alternatively, there may be greater availability of cell surface receptors or more susceptible cells for MNV in group 2 versus group 1 mice, so that the probability of ingested viruses binding to and entering host cells is much greater. Significant differences in susceptibility to the virus occurred between the mouse strains used in this study, suggesting that MNV transmission is dependent on the mouse strain exposed to the virus. With regard to MNV6, a difference in susceptibility to the virus between the 2 mouse strains was evident at the 3-wk time point. Thereafter, most of the pups in these litters were infected rapidly, suggesting that either this particular isolate replicates very rapidly in mice or that more virus was available in the feces excreted from infected parents and littermates. All of the fresh fecal pellets collected from pups exposed to MNV6 (and MNV5) after detection of MNV-positive fecal pellets contained live virus. Therefore, the pups were continuously seeding their cage with live virus and perpetuating environmental exposure to virus.

The distribution of MNV5 and MNV6 in the tissues of offspring mice from virus-exposed litters was similar between the mouse strains used in these experiments. From the perspective of molecular diagnostic testing, the ileum, cecum, and colon were the tissues infected most consistently in mice exposed to MNV5 or MNV6 and are therefore most likely to provide a positive diagnosis when mouse colonies are infected. In a recent study of adult mice inoculated with wildtype MNV,[11] the MLN and spleen (in addition to the intestine) were consistently positive for virus by RT-PCR. In the current study, MLN were consistently infected only in mice from groups 2 and 6 (NOD-scid × NOD/ShiLtJ). Whether virus susceptible cells are more abundant in this strain is not known. Few offspring showed positive RT-PCR results from splenic tissues. These differences in the tissue distribution between the mice in the current and previous[11] studies may be

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Table 5. Tissues of group 6 pups that were positive for MNV6 RNA by RT-PCR

Day	Duodenum	Jejunum	Ileum	Cecum	Colon	MLN	Liver	Spleen
14				X	X	X		
21	X	X	X	X	X	X		
24		X	X	X	X	X		
24		X	X	X	X	X		X
26		X	X	X	X	X	X	X
26	X	X	X	X	X	X	X	X
27	X	X	X	X	X	X		
27	X	X	X	X	X	X		
28	X	X	X	X	X	X		
28	X	X	X	X	X	X	X	
30	X	X	X	X	X	X		
30	X	X	X	X	X	X		
32	X	X	X	X	X	X		
32		X	X	X	X	X		
36	X	X	X	X	X	X	X	X
36	X	X	X	X	X		X	
36	X	X	X	X	X		X	
40	X	X	X	X	X	X	X	
40	X	X	X	X	X	X	X	
40	X	X	X	X	X	X	X	X

On each day, total RNA was isolated from the designated tissue and probed by specific primers for the presence of MNV6.

attributable to the age of the mice at the time of sampling and the strains of mice used, in addition to the tissue propensities of the wildtype viruses themselves. At this point, we know that groups 1 and 5 (NOD-scid) mice lack mature lymphocytes of both B-cell and T-cell lineages, whereas these cells are present in group 2 mice (NOD-scid × NOD/ShiLtJ). However, nothing in the literature suggests that MNV has tropism for lymphocytes. Possibly more relevant, another explanation for the difference in virus distribution to MLNs between mouse strains is the role that antigen-presenting cells play in MNV infections. The uptake and delivery of antigens, including infectious agents, from the gut to local secondary lymphoid tissues depends on the migration and accumulation of dendritic cells or macrophages (or both) from the lamina propria and Peyer patches of the gut to the MLNs after infection.[2,17] In immunocompetent mice, this migration of infected dendritic cells and macrophages delivers MNV directly to the MLN from infected gut tissues. The fate of these antigen-presenting cells in immunodeficient mice lacking competent lymphocytes is not known, but the low number of virus-positive MLN in scid mice suggests that in the absence of lymphocytes and the associated antigen presentation step, infected dendritic cells either leave the lymph node to migrate elsewhere or undergo apoptosis.

The 2 mouse strains used in this study to produce offspring differ in their capacities to mount an acquired immune response to a viral infection. Compared with mice on other genetic backgrounds, NOD-scid mice have a mutation in the *Prkdc* gene, and homozygotes rarely produce mature T and B lymphocytes and have a low probability of reverting to production of mature lymphocytes ('leakiness').[12] However, the NOD background confers additional immune dysfunctions, including defects in innate immunity, although these mice are capable of mounting an immune response to foreign antigens, including viruses.[18] In particular, mice on the NOD background are deficient in hemolytic complement, antigen presentation by macrophages,[27] macrophage cytokine elaboration,[7,9] natural killer and natural-killer T cell numbers and function[13,28] and defects in the T cell immunoregulatory network.[4] Taken together, these deficiencies are sufficient to make NOD/ShiLtJ mice readily susceptible to viral infections. Nonetheless, N1 NOD-scid × NOD/ShiLtJ mice, which failed to clear the virus or stop virus shedding in this study, produced antibodies to MNV in response to infection and survived the infections. This finding is somewhat surprising, given the inherently reduced numbers of NK cells, which are critical to the early, innate immune defense against viral infections.

NOD/ShiLtJ mice are valued as a research model of type 1 (autoimmune) diabetes.[18] However, both experimental[9,26] and natural[32] viral infections of NOD/ShiLtJ mice result in a decline in the percentage of animals that develop autoimmune type 1 diabetes. Recent studies with mouse rotavirus in NOD/ShiLt mice[8] further confirmed this effect of viral infection on development of autoimmune diabetes in NOD mice and associated the effect with the dissemination of viral antigens to extraintestinal tissues, including the pancreas. This alteration of the diabetic phenotype has been hypothesized to result from interference in the antiβ cell immune response because of a shift in the immunoregulatory cytokine cascade induced by the viral infection. For this reason NOD/ShiLtJ breeding colonies require high barrier maintenance to prevent loss of phenotype. Currently, we are examining whether MNV infection alters the NOD/ShiLtJ diabetic phenotype.

Identification of sequence differences between the *Orf2* genes of MNV isolates from infected breeding mice and their offspring is of great interest. It is not surprising that we found nucleotide sequence changes and corresponding changes in predicted amino acids between the 2 generations of virus obtained after passage from parental to offspring mice. Mutations in the nucleotide sequence of MNV should be expected, given that single-stranded

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Table 6. Serologic responses of offspring and male breeding mice exposed to MNV5 and MNV6

		No. of mice	Results
Group 2			
Offspring			
	3-wk exposure to infected dam	2	all –
	4-wk exposure to infected dam	2	all –
	5-wk exposure to infected dam	7	all –
	6-wk exposure to infected dam	5	+,+,–,+,+
	7-wk exposure to infected dam	3	+,+,+
Breeders			
	week 3 after exposure	2	+,+
	week 27 after exposure	2	+,+
Group 6			
Offspring			
	4-wk exposure to infected dam	7	all –
	5-wk exposure to infected dam	1	all –
	6-wk exposure to infected dam	6	+,+,–,+,+,+
Breeders			
	week 17 after exposure	2	+,+

Sera from random offspring and male breeders from immunocompetent experimental breeding colonies (groups 2 and 6) were tested for anti-MNV antibodies.

Table 7. Changes in nucleotide and predicted amino acid sequences of *Orf2* in MNV5 and MNV6 isolated from breeding mice and their offspring

Nucleotide sequence		Amino acid sequence	
Position	Nucleotide (Breeder-Offspring)	Position	Amino acid (Breeder/Offspring)
MNV5			
99	G/A	33	Gln/Gln
235	C/ T	78	Leu/Leu
282	T/ C	94	His/His
390	C/T	130	Tyr/Tyr
414	C/T	138	Thr/Thr
426	A/G	142	Thr/Thr
678	T/C	226	Ile/Ile
969	A/G	323	Ala/Ala
996	T/C	332	Ser/Ser
1017	C/T	339	Val/Val
1023	C/T	341	Thr/Thr
1062	T/C	354	Thr/Thr
1072	A/G	357	Ile/Val
1094	C/T	364	Ala/Val
1107	C/T	369	Pro/Pro
1118	G/A	372	Arg/Lys
1135	A/G	378	Thr/Ala
1137	T/C	379	Thr/Ala
1149	T/C	383	Ser/Ser
1156	T/C	385	Leu/Leu
1527	C/T	509	Phe/Phe
MNV6			
150	G/A	50	Gln/Gln
849	T/C	283	Gly/Gly
902	T/C	300	Ile/Thr
1121	T/C	373	Val/Ala
1620	G/A	540	Lys/Lys

RNA viruses are noted for spontaneous mutations in the genome and have a comparatively high mutation rate, compared with DNA-containing organisms.[5,6,10] Like other single-stranded RNA viruses, murine noroviruses are prone to errors in the replication of their RNA genomes because of poor proofreading functions in the RNA-dependent RNA polymerase.

Although deficiencies in the proofreading capabilities of viral RNA polymerase likely were a major driving force for the initiation of mutations in the MNV genome, another potential cause of the observed number of nucleotide changes we report here could be the presence of coinfecting viruses. We did not plaque-purify MNV5 and MNV6 prior to use in these experiments because our primary consideration was to maintain their 'wildtype' phenotype and because others have demonstrated that passage of MNV in cell culture can result in an accumulation of mutations.[33] However, our nucleotide sequencing of parental viruses isolated from breeding mice did not show any ambiguous results or overlapping peaks and therefore suggested that we indeed were dealing with single virus isolates. The important question pertaining to the observed genomic substitutions with regard to MNV is whether they result in biological differences in the progeny virus that emerge from infected animals. This answer remains to be determined for MNV in general, because so few isolates have been studied to date. Nevertheless, a single amino acid change in the MNV VP1, P2 domain was sufficient to prevent virus neutralization,[20] and mutations clustered in the same region have been found in human calicivirus.[24] Furthermore, as mentioned earlier, in vitro passage of MNV1 resulted in the accumulation of nucleotide and corresponding amino acid changes that led to loss of virulence of the progeny viruses produced in cell culture.[33]

Murine noroviruses may be amenable to genotyping by means of the *Orf2* region, as is done for human noroviruses.[1,30] This would aid the laboratory animal community in mapping specific MNV genotypes within animal facilities. Because *Orf2* encodes the outer capsid protein, which is a predominant target of the immune response[19,23] use of this gene allows correlation of sequencing and serologic data. Genomic variations in different MNV isolates can then be linked directly to differences in virus biology, including pathogenesis.

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