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Indirect ELISA and Indirect Immunofluorescent Antibody Assay for Detecting the Antibody against Murine Norovirus S7 in Mice

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Abstract: To evaluate murine norovirus (MNV) infection in laboratory mice, we attempted to develop an enzyme-linked immunosorbent assay (ELISA) system and an indirect immunofluorescent antibody (IFA) assay for detecting the anti-MNV-S7 antibody in mice. MNV-S7, which was isolated in Japan, was used in both assays. The antigen for ELISA was prepared by ultracentrifugation of culture supernatants of RAW 264 cells infected with MNV-S7. Positive sera were obtained from 6-week-old, female C57BL/6JJcl mice inoculated orally with MNV-S7. IFA against infected RAW 264 cells was able to discriminate positive sera from negative sera. Indirect ELISA was performed using 96-well ELISA plates coated with formalin-treated MNV-S7 antigen. In this ELISA system, mouse sera obtained 2 weeks after infection or later showed significantly high OD values and were judged positive. An equal level of anti-MNV-S7 antibody response was observed in BALB/cAJcl, C57BL/6JJcl, DBA/2JJcl, and Jcl:ICR mice; whereas, C3H/HeJJcl mice demonstrated slightly lower antibody production 4 weeks after infection. We also used this ELISA system to evaluate 77 murine serum samples obtained from 15 conventional mouse rooms in research facilities in Japan and found that approximately half of the serum samples contained antibody to MNV-S7. We found that some serum samples were negative for antibodies to mouse hepatitis virus and *Mycoplasma pulmonis* but positive for antibody to MNV-S7. The results suggest that the MNV infection is more prevalent than other infections such as mouse hepatitis virus and *Mycoplasma pulmonis* in conventional mouse colonies in Japan, as is the case in other areas of the world.

Key words: enzyme-linked immunosorbent assay, immunofluorescent antibody assay, murine norovirus

Introduction

The murine norovirus (MNV) was recently isolated from immunodeficient mice that succumbed to a disease of unknown etiology [9, 23]. Genetic analysis of this agent has revealed that the pathogen (MNV-1) is close-

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ly related to human norovirus, a member of the *Caliciviridae* family of positive-strand RNA viruses. In addition to MNV-1, several MNV strains have been isolated from laboratory mice [6, 10, 20]. Generally, MNV infection does not show any clinical symptoms in immunocompetent mice [23], except for occasional association with histopathological changes in the intestine and the spleen [14]. On the other hand, it sometimes induces noticeable symptoms such as diarrhea in immunodeficient mice, such as OTI/Rag1$^{-/-}$IFN-$\gamma^{-/-}$ and β_2M$^{-/-}$ mice [20]. Persistent MNV infection has been reported in not only immunodeficient but also immunocompetent mice [4, 6, 14]. However, viral persistence is a viral strain-dependent phenomenon, since Karst *et al*. demonstrated that viral RNA was no longer present in visceral and mucosal tissues collected from immunocompetent mice 3 days after oral infection with MNV-1 [9]. Although MNV exhibits biological diversity as stated above, it has been found to comprise a single genogroup as well as a single serotype [20].

A recent study of a large number of mouse serum samples from research colonies in the United States and Canada identified MNV-1 reactive antibodies in 22.1% of serum samples [7]. In addition, the results of a serological survey that evaluated the contemporary prevalence of MNV in laboratory mice in Western Europe indicated that MNV is the most prevalent viral pathogen [13]. Although Goto *et al*. recently demonstrated MNV prevalence in Japan by RT-PCR [4], there is no information available on the MNV prevalence in laboratory mouse colonies in Japan based on serology.

In this study, an indirect immunofluorescent antibody (IFA) assay and an ELISA system were developed using MNV-S7, a new MNV strain that was isolated in Japan. The results suggest that MNV infection is more prevalent than mouse hepatitis virus (MHV) and *Mycoplasma pulmonis* (*M. pulmonis*) infections in conventional mouse colonies in Japan, as is the case in other areas of the world [13, 15].

Materials and Methods

Mice and sera

Female, 6-week-old specific pathogen-free C57BL/6JJcl (B6), BALB/cAJcl, C3H/HeJJcl, DBA/2JJcl, and Jcl:ICR mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The breeding colonies except for Jcl:ICR were free of MNV when examined by RT-PCR (Dr. Goto, personal communication).

Two experimental MNV-S7 infections of mice were carried out. The first one was a time course study using B6 mice. The second one was a strain difference study using five strains of mice. Mice were orally inoculated with 0.1 ml of 5×10^5 $TCID_{50}$ of MNV-S7 on the day when they arrived at our laboratory. They were housed in isolation racks in a room maintained at a temperature of 23°C on a 12:12-h light-dark cycle, and were fed on commercial pellet CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. After being anesthetized with isoflurane, blood was collected from naive and MNV-S7-infected mice by cardiac puncture. Sera were collected and stored at –80°C until use. In the time course study, 4, 3, 3, 4, 4, and 2 B6 mice were sacrificed at 1, 2, 3, 4, 5, and 6 weeks after infection, respectively. Five naive B6 mice were used as negative controls. In the strain difference study, four each of B6, BALB/cAJcl, C3H/HeJJcl, DBA/2JJcl, and Jcl:ICR mice were sacrificed at 4 weeks after infection. Five or four naive mice of each mouse strain were used as negative controls. The animal experiments were approved by the institutional animal care and use committee of the University of Tokyo and carried out in accordance with the guidelines for animal experimentation of the University of Tokyo, Japan.

Sera from mice bred in conventional animal facilities were obtained from several universities and research institutions in Japan. Antibodies to MHV and *M. pulmonis* were examined by the use of MONILISA kits (Wakamoto Pharmaceutical, Tokyo, Japan) in accordance with the manufacturer's instructions.

Virus and cells

MNV-S7, which was recently isolated from a conventional mouse in Japan (Genbank accession no. AB435515), was used. RAW 264, a macrophage-like tumor cell line was obtained from the RIKEN BioResource Center Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.1 mM non-essential amino acids, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The virus was

propagated and titrated in RAW 264 cells using a conventional 50% tissue culture infectious dose ($TCID_{50}$) assay. Briefly, viral suspensions were five-fold serially diluted (5^{-1} to 5^{-10}) in 100 μl of DMEM medium to inoculate eight wells per dilution in 96-well plates, and a suspension of RAW 264 cells was added to each well (total volume of 200 μl/well). The cultures were maintained at 37°C, 5% CO_2, and after 2 days, the cytopathic effect was manually recorded. $TCID_{50}$/ml values were calculated according to the Reed and Muench method [16], where the tissue culture infectious dose is the virus dilution at which 50% of the wells contained infected cells. The results are presented as the –log mean of 4 replicates for each measured virus concentration.

Indirect immunofluorescent antibody (IFA) assay

RAW 264 cells were cultured in chamber slides (Nalge Nunc International, Rochester, New York) and inoculated with MNV-S7 at a multiplicity of infection (MOI) of 0.1. Eighteen to 20 h later, the infected cells were fixed in 4% paraformaldehyde and 8% sucrose in PBS for 20 min and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. The cells were treated with 5% bovine serum albumin (BSA) in PBS for 30 min at room temperature and incubated with diluted mouse serum in PBS containing 1% BSA for 1 h at room temperature. After being washed three times, the cells were incubated with Alexa Fluor 488-conjugated anti-mouse IgG (H and L chain)(Molecular Probes, Eugene, Oregon) diluted to 1:500 in PBS for 1 h at room temperature. The samples were then analyzed using a confocal laser-scanning microscope (LSM510 Version 2.02; Carl Zeiss, Jena, Germany).

Preparation of MNV-S7 antigen for ELISA

RAW 264 cells were inoculated with MNV-S7 at an MOI of 0.1 and then cultured in DMEM supplemented with 10% FCS in 150 mm dishes for 40–48 h. The culture supernatant was collected and centrifuged at 2,000 rpm (800 × *g*) for 10 min at 4°C to remove cellular debris. The resultant supernatant was collected and the viral titer was determined. One hundred and eighty milliliters of the supernatant that showed $10^{5.83}TCID_{50}$/0.1 ml were centrifuged at 25,000 rpm (105,000 × *g*) for 90 min in an SRP-28SA rotor (Hitachi Koki, Tokyo, Japan). The resultant pellets were resuspended in TN buffer (0.01 M Tris-HCl and 0.1 M NaCl, pH 8.0) and then layered on top of a 1-ml 30% sucrose cushion and centrifuged at 25,000 rpm for 90 min in the SRP-28SA rotor. The viral pellets were dissolved with 1.8 ml TN buffer containing 0.1% sodium dodecyl sulfate. After incubation at room temperature for 10 min, the suspension was centrifuged at 1,000 × *g* for 15 min at 4°C. The supernatant ($10^{6.61}$ $TCID_{50}$/0.1 ml) was collected, divided into samples, and stored at –80°C until use. These procedures succeeded in achieving a concentration that was approximately 30 times higher than that estimated from the infectious viral titers. Purified virus was treated with 0.1% formalin for a week at room temperature, dialyzed against PBS at 4°C, and used as a viral antigen for ELISA. To examine the presence of infectious virus in the antigen, the antigen was filtrated with a 0.45-μm membrane filter and inoculated into RAW 264 cells, and the cytopathic effect was monitored. To further confirm the absence of infectious virus in the antigen, supernatant collected from RAW 264 cells that had been blindly passaged three times with the antigen was examined using the IFA assay.

ELISA

Aliquots (100 μl) of the MNV-S7 antigen diluted 1:100 in carbonate coating buffer (0.032 M Na_2CO_3, 0.068 M $NaHCO_3$, pH 9.6) were added to each well of a 96-well plate (Thermo Fisher Scientific, Roskilde, Denmark) and allowed to incubate at 4°C overnight. After being washed three times with Tris-buffered saline (TBS) containing 2% Tween 20, the wells were incubated with carbonate coating buffer containing 5% skimmed milk (blocking buffer) at room temperature for 1 h. After being washed three times, 50 μl of mouse sera diluted with blocking buffer was added to each well and incubated at 37°C for 1 h. After being washed three times, horseradish peroxidase-conjugated rabbit anti-mouse IgG (γ chain) antibody (ZYMED, San Francisco, California) diluted 1:2,000 in PBS was added to each well and incubated at 37°C for 1 h. After being washed four times, 100 μl of SureBlue™ TMB substrate (KPL, Gaithersburg, Maryland) was added and incubated at room temperature for 10 min. The reaction was stopped by adding 100 μl of 1 M H_2SO_4, and the OD_{450} was determined with a Wallac 1420 ARVOsx plate reader

(PerkinElmer, Waltham, Massachusetts). To inhibit contamination by liquid waste from the ELISA system, we autoclaved all the liquid waste and the ELISA plates.

Statistics

Statistical analysis was performed using Student's *t*-test and the chi-square test, with $P<0.05$ considered significant.

Results

IFA assay to detect the anti-MNV-S7 antibody in mice

Five, naive B6 mice were sacrificed, and their sera were used as a negative control. Twenty B6 mice were orally inoculated with 5×10^5 $TCID_{50}$ of MNV-S7, and 2 to 4 mice were sacrificed each week for sera collection. The sera of uninfected and infected mice were used in the IFA assay to detect anti-MNV-S7 antibody. The sera of the naive B6 mice did not show immunofluorescence after incubation with MNV-S7-infected RAW 264 cells. On the other hand, the sera obtained from MNV-infected mice fluoresced vigorously, and the antibody titers increased with time (Fig. 1). It is worth noting that the mouse sera obtained at 1 week postinfection showed a relatively high antibody titer in the IFA assay. As shown in Fig. 2, we found immunofluorescence in the cytoplasm of MNV-infected RAW 264 cells.

Establishment of ELISA to detect the anti-MNV-S7 antibody in mice

Before establishing the ELISA system, we examined the presence of infectious virus in purified MNV-S7 antigen. RAW 264 cells that had been inoculated with the antigen did not show a cytopathic effect, suggesting the absence of infectious virus in the antigen. To confirm the absence of infectious virus in the antigen, supernatant collected from RAW 264 cells that had been blindly passaged three times with the antigen was examined using the IFA assay. Unexpectedly, we found weak immunofluorescence in the cytoplasm of the inoculated RAW 264 cells, suggesting that a small amount of infectious virus still remained in the antigen. To inhibit contamination from our ELISA system, we decided to autoclave all the liquid waste and the ELISA plates.

To construct an ELISA system for detecting the anti-MNV antibody in mice, we carried out a box titration. Purified MNV-S7 antigen was serially diluted and added to the wells of a 96-well plate for adsorption, then a B6 mouse serum obtained at 6 weeks postinfection (IFA titer 1:12,800) was also serially diluted and added to the wells, and enzymatic action was developed according to the procedure described in Materials and Methods (Fig. 3). The OD_{450nm} value generally decreased along with serum dilution, and the OD_{450nm} value induced by the antigen diluted to 1:50 was lower than that calculated for the antigen diluted to 1:100. The highest OD_{450nm} value was observed when the MNV-S7 antigen was diluted to 1:100 and the serum was diluted to 1:100. From these results, we adopted 1:100 diluted MNV-S7 antigen

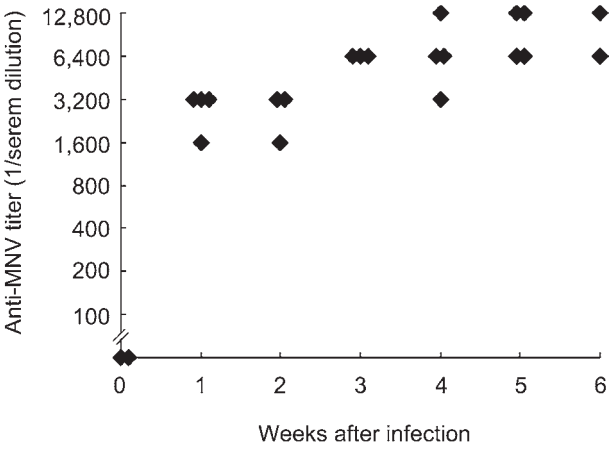

Fig. 1. IFA reactivity of sera from naive and experimentally MNV-S7-infected B6 mice. Three to five mice were sacrificed each week, and antibody to MNV-S7 was examined by IFA.

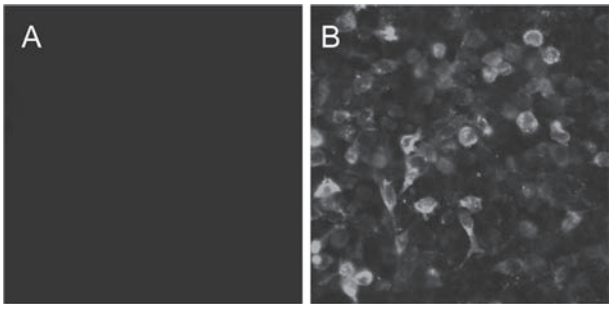

Fig. 2. Photographs of the IFA assay. RAW 264 cells infected with MNV-S7 were incubated with the sera of naive (A) and experimentally MNV-S7-infected B6 mice (B) and were then treated as described in Materials and Methods.

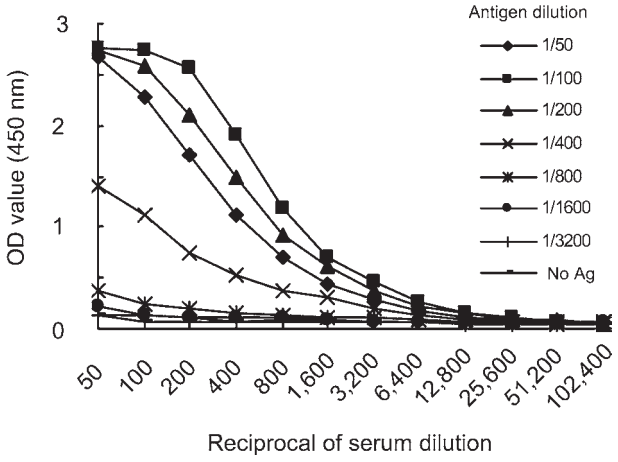

Fig. 3. ELISA reactivity of serially diluted MNV-S7 antigen and an experimentally MNV-S7-infected B6 mouse serum (Box titration).

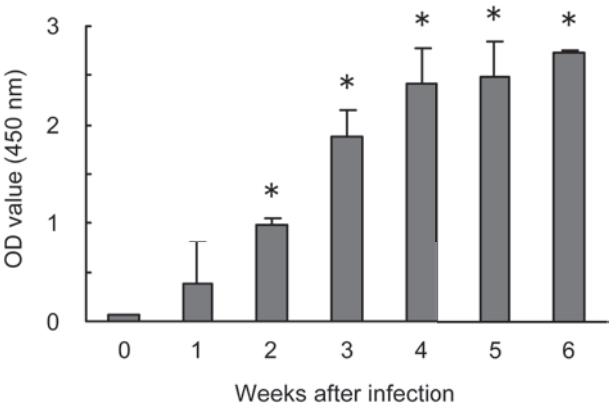

Fig. 4. ELISA reactivity of sera from naive and experimentally MNV-S7-infected B6 mice. Two to five mice were sacrificed each week, and antibody to MNV-S7 was examined by ELISA (OD450 nm) (Mean ± SD). *: significantly different from naive mice (P<0.05).

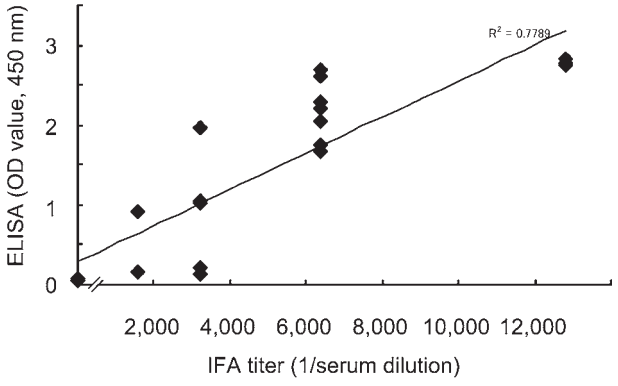

Fig. 5. Comparison of ELISA and IFA results in naive and experimentally MNV-S7-infected C57BL/6JJcl mice. ELISA (OD450 nm) and IFA (1/serum dilution) titers are plotted. (R^2=0.7789, P<0.05).

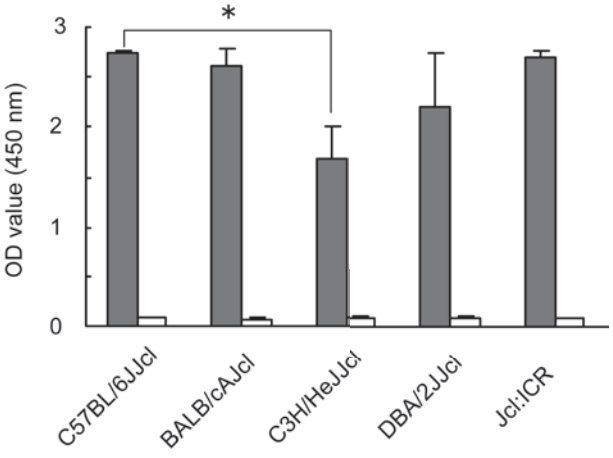

Fig. 6. ELISA reactivity of sera from four inbred strains and ICR mice infected with or without MNV-S7. Sera were obtained from naive BALB/cAJcl, B6, C3H/HeJJcl, DBA/2JJcl, and Jcl:ICR mice and those orally inoculated with MNV-S7 4 weeks ealier and were assayed using the anti-MNV antibody ELISA. Each group consisted of 4 or 5 mice. The grey columns represent infected mice (Mean ± SD), and the open columns represent naive mice (Mean ± SD). *: P<0.05.

and mouse sera diluted to 1:100 for our ELISA system for detecting the antibody to MNV-S7.

Twenty-five B6 mouse sera, whose anti-MNV-S7 antibody titer had been measured by the IFA assay, were assayed using our ELISA system (Fig. 4). The OD_{450nm} values of naive mouse sera were around or less than 0.1. Although the sera obtained at 1 week postinfection showed a weak response, the response was not significantly different from that of the naive mice. A significantly positive response was observed in the sera collected at 2 weeks postinfection or later, and the OD_{450nm} value generally increased with time. The antibody response judged by ELISA was correlated with the titer measured by the IFA assay (R^2=0.7789, P<0.05) (Fig. 5).

To examine whether this ELISA system was useful for other strains of mice, sera from B6, BALB/cAJcl, C3H/HeJJcl, DBA/2JJcl, and Jcl:ICR mice (4 weeks postinfection) with or without MNV-S7 infection were applied to our ELISA system (Fig. 6). Although all the

Table 1. Anti-MNV-S7 ELISA titer in mice bred in conventional animal facilities

Room	OD value (450 nm)			
	0–0.149	0.15–0.499	0.5–0.999	1–
A				8/8[a)]
B				4/4
C	3/3			
D	3/4	1/4		
E	3/3			
F	1/2	1/2		
G		3/3		
H				10/10
I				3/3
J				7/7
K	1/6			5/6
L	8/10	2/10		
M				4/4
N		6/8		2/8
O				2/2

[a)]No. of samples with indicated OD value/Total sample numbers of each room.

Table 2. Summary of anti-MNV, anti-MHV, and anti-*M. pulmonis* antibodies in murine serum samples from conventional animal facilities in Japan

Animal facilities	Antibodies to		
	MNV	MHV	*M. pulmonis*
A	8/8 (100%)[a)]	8/8 (100%)	8/8 (100%)
B	4/4 (100%)	4/4 (100%)	0/4 (0%)
C	0/3 (0%)	0/3 (0%)	0/3 (0%)
D	0/4 (0%)	0/4 (0%)	0/4 (0%)
E	0/3 (0%)	0/3 (0%)	0/3 (0%)
F	0/2 (0%)	2/2 (100%)	0/2 (0%)
G	0/3 (0%)	0/3 (0%)	0/3 (0%)
H	10/10 (100%)	10/10 (100%)	10/10 (100%)
I	3/3 (100%)	0/3 (0%)	0/3 (0%)
J	7/7 (100%)	7/7 (100%)	0/7 (0%)
K	5/6 (83.3%)	0/6 (0%)	0/6 (0%)
L	0/10 (0%)	0/10 (0%)	0/10 (0%)
M	4/4 (100%)	4/4 (100%)	4/4 (100%)
N	2/8 (25%)	2/8 (25%)	2/8 (25%)
O	2/2 (100%)	0/2 (0%)	0/2 (0%)
Total	45/77(58.4%)	37/77(48.1%)	24/77(31.2%)

[a)]No. of positive samples/No. of total samples (%).

strains of mice tested mounted a significant antibody response during MNV-S7 infection, the OD_{450nm} value of the C3H/HeJJcl mice was significantly lower than that of the B6 mice. It is worth noting that no nonspecific responses were induced in uninfected mouse sera.

Antibodies to MNV-S7 in mice bred in conventional animal facilities in Japan

Seventy-seven mouse sera from 15 conventional animal facilities (A to O) in Japan were obtained and assayed using our ELISA system for detecting anti-MNV-S7 antibody (Table 1). Of the 77 samples tested, the OD_{450nm} values of 19 samples (24.7%) were less than 0.15, those of 13 samples (16.9%) were between 0.15 and 0.5, and those of 45 samples (58.4%) were over 1.0. The OD_{450nm} values of all the mouse sera from two conventional animal facilities (C and E) were less than 0.15. On the other hand, those of mouse sera from seven other conventional animal facilities (A, B, H, I, J, M, and O) were over 1.0. Thirteen mouse sera that showed a weak response in ELISA (0.15–0.499) were further tested using the IFA assay. While eight sera diluted to 1:50 were found to be negative for MNV by IFA, five mouse sera diluted to 1:50 were shown to be weakly positive (data not shown). A cutoff value of 0.3 OD_{450nm} was tentatively set for the ELISA, which minimized the inconsistency between the results of the IFA assay and those of ELISA. With this benchmark, 1 week postinfection results gave an inconsistent assessment between the IFA assay and ELISA for three B6 sera from 67 sera (including negative controls) with experimental MNV-S7 infection in this study (Fig. 5).

In addition to antibody to MNV, the mouse serum samples were examined for antibodies to MHV and *M. pulmonis* using a commercial ELISA kit. As shown in Table 2, MNV was most prevalent (58.4%) among these three murine pathogens. It is worth noting that sera from some animal facilities (I, K, and O) were positive only for MNV.

Discussion

In the present study, we developed serological assay systems (ELISA and IFA) for MNV infection in mice using MNV-S7, which was isolated in Japan, and applied them to murine sera from conventional animal facilities in Japan. Serological tests are the most common methodology used in viral surveillance of laboratory mouse colonies. They include the complement fixation test, hemagglutination inhibition test, neutralization test,

ELISA, and IFA. The last two methods are inexpensive, rapid, sensitive, and specific, and therefore, are used widely all over the world [2, 3, 18, 19]. Since ELISA systems performed in a 96-well plate format are suitable for handling large numbers of samples, they are frequently used in screening tests. Samples that are shown to be positive in ELISA tests usually undergo an IFA test to confirm the results. Some ELISA kits for detecting antibodies to rodent viruses are commercially available and are used in in-house examinations. In the present study, we used a commercially available kit to examine antibodies to MHV and *M. pulmonis*. Moreover, viral antibody responses are detected for prolonged periods even in the recovery stage after acute infection. Therefore, serological examination allows us to retrospectively check the microbiological status of laboratory mouse colonies. These merits of serological tests have made them the standard diagnostic measures for viral infections in laboratory mice, even though molecular detection techniques have been developed for most viral infections [4, 21]. An important caveat is that serology is invalid for mice that have just been infected and immunodeficient mice, which cannot produce immunoglobulins. In these cases, a molecular detection technique such as PCR/RT-PCR is efficacious. A microsphere-based, multiplexed fluorescent immunoassay has been recently developed as a high-throughput, automated screening test to complement traditional ELISA in laboratory animal medicine [7, 15].

The antibody response judged by ELISA was correlated with the titer measured by the IFA assay during acute MNV-S7 infection. However, we found a minor inconsistency. Three of four B6 sera at 1 week postinfection were judged negative at a cutoff value of 0.3 by ELISA, whereas all four sera were judged positive by IFA assay. Although the cutoff value was tentatively set at 0.3 to minimize the inconsistency between the results of the IFA assay and those of ELISA for sera from conventional animal facilities, a lower cutoff value might be suitable when ELISA is used for screening. Serum samples showing an OD value above 0.121 (Mean + 3 SD in negative controls) by ELISA should be examined by IFA assay, and a few weeks later serum samples obtained from the same mouse colony should be checked again.

In this study, we demonstrated that the prevalence of MNV was higher than those of MHV and *M. pulmonis*, which was consistent with serological survey data that indicates that MNV is the most prevalent viral pathogen in contemporary mouse colonies [13, 15]. We found that some serum samples were negative for antibodies to MHV and *M. pulmonis* but positive for antibody to MNV-S7. Therefore, we should not suppose that mice negative for antibodies to MHV, Sendai virus, and *M. pulmonis*, which are commonly examined in serological tests in Japan, are also clear of MNV.

We were surprised at the high prevalence rate of MNV infection found in this study, which we partly attribute to the limited number of samples. Judging from the data concerning MHV and *M. pulmonis* infection, the sera used in this study may have included sera from mice bred under substandard microbiological controls. Recently, Goto *et al*. reported the epidemiology of MNV infection in laboratory mice in Japan using RT-PCR [4]. They detected the MNV gene from 33/245 (13.1%) cecal samples derived from 15/59 (25.4%) facilities. The samples used in the two studies were different, and the detection methods were also different: RT-PCR detects viral RNA, whereas ELISA measures specific antibodies produced *in vivo*. Since some MNV strains may induce an antibody response without viral RNA persistence in mice, as MNV-1 does even though the precise mechanism remains unknown [9], it is possible that the MNV prevalence assessed by serological tests is higher than that estimated by RT-PCR.

Noroviruses have been shown to be relatively resistant to physical and chemical inactivation [5]. In this study, purified MNV-S7 was treated with 0.1% formalin for a week at room temperature and then used as an antigen for coating ELISA plates. Contrary to our expectation, a small amount of infectious virus still remained in the antigen. To inhibit contamination from our ELISA system, we autoclaved all the liquid waste and the ELISA plates. It need scarcely be said that the data were not corrupted by the presence of infectious virus in the MNV antigen. However, the use of completely inactivated virus or a recombinant protein [19] that lacks a contamination risk may be preferable.

Several studies have indicated that resistance against viral infections is dependent on mouse strain [1, 8, 9, 11,

17]. Namely, numerous host factors, both major histocompatibility complex (MHC)-dependent and MHC-independent, account for viral resistance. In this study, we demonstrated that the antibody level of C3H/HeJJcl mice was significantly lower than that of C57BL/6JJcl mice. To investigate the cause of the difference, we will compare MNV-S7 replication in these strains of mice as antibody production is influenced by the dose of antigen loaded *in vivo*. Further studies may reveal factors that affect the replication of MNV.

Periodic health screening of laboratory rodents is necessary due to the adverse effect of infectious diseases on biomedical research. However, the influence of MNV infection on animal research remains obscure. Most reports have stated that MNV infection in immunocompetent mice was asymptomatic [4, 6, 9, 23]. In this study, we also observed C57BL/6 mice inoculated with MNV-S7, and they did not show any clinical signs (data not shown). However, it has been recently shown that concurrent MNV infection accelerates the progression of *Helicobacter bilis*-induced inflammatory bowel disease in Mdr1a$^{-/-}$ mice [12]. Since MNV infects dendritic cells and macrophages [22], it is likely that MNV induces immune system disorders. Further study is needed to understand the influence of MNV infection on animal research.

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