Studies on control measures for viral contamination in a laboratory animal facility

（動物実験施設におけるウイルス汚染の防除対策に関する研究）

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General Introduction

Contamination of laboratory animals with pathogenic microorganisms affects the health of animals and may cause misleading the results of animal experiments. High microbiological quality is therefore required for laboratory animals especially for those used in biomedical science researches. Quite a few microorganisms have been eliminated from the breeding colonies of laboratory animals to establish specific pathogen free (SPF) animals.

SPF animals are normally kept in barrier facilities which are equipped with necessary functions, such as ventilation, sterilization, and housing systems, for the maintenance of cleanliness level. However, there are still risks of microbiological contamination depending on the way of management, even when barrier systems work well. In this paper, I focus attention on three issues regarding control measures in management of a laboratory animal facility for microbiological contamination, and show the results of some cases of viral contamination.

The first issue is “microbiological monitoring”. To keep cleanliness of the facility, only SPF animals should be introduced from breeders or other animal facilities. In addition, animals in the facility should be SPF during rearing period. Microbiological monitoring is a measure for confirming that they are SPF. Traditional methods are cultivation of bacteria, microscopic examination of parasites, and serological tests for viruses and bacteria, such as enzyme-linked immunosorbent assay (ELISA), complement fixation (CF) assay, and

immunofluorescence assay (IFA). Recently, multiplexed fluorometric immunoassay (MFIA) is also developed for serological tests. For detecting the genomes of contaminated microorganisms, polymerase chain reaction (PCR) tests for various agents have been also developed (Furukawa *et al*., 2002, Kuramochi *et al*., 1997, Nozu *et al*., 1999, Yamada *et al*., 1993).

In serological tests, ELISA is usually performed to screen for viruses and some bacteria, because the sensitivity is high and many samples can be handled at one time. Some commercial ELISA kits for murine pathogens are available. IFA is mostly used as a confirmatory test for sera judged as positive by ELISA, because the specificity is high, but special techniques and equipments are required, such as making antigen slides of pathogens and fluorescent microscope. Most antigens used for ELISA are generally prepared by multiplying viruses in cells (Buller *et al*., 1983, Kagiyama *et al*., 1990, Turkovic and Ljubicic, 1992). Recombinant antigen expressed in *Escherichia coli* is also developed for the virus that is difficult to grow in cells (Kunita *et al*., 2006). Compared to antigen prepared from cells, recombinant antigen has an advantage of using no infectious virus during preparation of antigen. Because some viruses are very contagious and there is a potential risk of contamination in a laboratory and an animal facility in using infectious virus, it is desirable not to use infectious virus during preparation of antigen, especially of zoonotic virus like

lymphocytic choriomeningitis virus (LCMV). Recently, baculovirus expression vector system has been used as a useful method for recombinant protein expression. This system provides high levels of recombinant gene expression obtained with the viral polyhedrin promoter. Biologically active and functional recombinant proteins are produced by the eukaryotic posttranslational modification in insect cells. Besides, they can be used under lowest physical containment system in a laboratory, since baculoviruses are essentially nonpathogenic to mammals including humans. Matsuura *et al*. (1987) made recombinant LCMV-nucleoprotein (NP) antigen by the use of baculovirus expression vector system. In Chapter 1, investigation of the utility of ELISA system using recombinant LCMV-NP antigen for detecting anti-LCMV antibody was conducted for recently isolated strain and a well-documented laboratory strain of LCMV.

Even though the management has been well conducted, many facilities have experienced microbiological contamination through unpredictable routes. The second issue is “detection of the origin of contamination”. In the National Institute of Infectious Diseases Japan (NIID), only SPF animals have been allowed to be introduced to a barrier area in the animal facility from commercial breeders or other institutions. The microbiological qualities of laboratory animals including mice, guinea pigs, and rabbits have been examined monthly by regular microbiological monitoring in the NIID. Even though barrier environment was

maintained at the NIID facility under these management systems, accidental contaminations of rabbits with *Bordetella bronchiseptica*, mice with mouse hepatitis virus (MHV) and Sendai virus (HVJ) occurred in the 1990's (Takimoto *et al*., 1999). The contamination of *B. bronchiseptica* was caused by rabbits infected with *B. bronchiseptica* introduced from a commercial breeder. MHV contamination occurred in mouse breeding colonies. In this case, the source of MHV contamination was unfortunately unclear, because mouse colonies from different institutions were maintained in an animal room over a period of time and most colonies were MHV positive when the contamination was revealed. HVJ contamination in mice occurred during animal experiment using a mouse-adapted influenza virus. In Chapter 2, detailed reports on the investigation of the origin of this HVJ contamination and the elimination of HVJ from the contaminated virus stock are described.

The third issue is "cleaning and disinfection". In animal facilities, cleaning and disinfection should be routinely performed to provide a healthy environment for laboratory animals. Currently, researchers can commercially obtain many types of disinfectants, such as alcohols, aldehydes, chlorine, oxidizing agents, phenols, iodine, quaternary ammonium compounds and so on. The properties are different among these disinfectants, so it is necessary to select suitable agent for each animal facility depending on its own environment to be maintained. It is important for disinfectants used in animal facilities not only to exert

high disinfection efficacy but also to be as safe as possible for animals and personnel. Because disinfectants are usually used by all the personnel in an animal facility, they are also required to be easily used, like using without dilution, and minimal undesirable nature, such as odor, irritant properties, staining properties, and corrosiveness. In addition, cost effectiveness plays a key role in the selection of disinfectant. Recently, weak acid hypochlorous solution (WAHS) is brought into use as one of main disinfectants in animal facilities, hospitals, and food industries due to some advantages such as its high antiseptic efficacy, low cost, and safety for animals and humans. Another important advantage of WAHS is that it can be used as disinfectant and as drinking water without dilution. The efficacy of WAHS against some bacteria and viruses of laboratory animals were reported by the laboratory I belong to (Taharaguchi *et al*., 2014). In Chapter 3, I evaluated the efficacy of WAHS against murine norovirus (MNV) and compared the efficacy with diluted NaOCl (Purelox) and 70% ethanol that are commonly used in animal facilities. In addition, taking advantage of WAHS to be able to drink, I attempted to evaluate the ability of WAHS supplied as drinking water for eliminating and preventing MNV infection.

Accidental contaminations with various microorganisms have occurred in laboratory animal facilities. Recently, many laboratory animals are introduced from overseas to use for research. Due to the increase of the use of these mice, the necessity of measures for

microorganisms hardly found in Japan becomes higher. The transfers of genetically modified mice, such as transgenic and knockout mice, are also increased between animal facilities (Yamamoto, *et al*., 2001). Because most of these animals are generated in each animal facility of universities and research institutions and supplied to other animal facilities, the possibility of microbiological contamination of these animals is considered to be higher than that of animals bred in the facility of commercial breeders (Shek, 2008). Therefore, control measures using more precise microbiological test and more effective disinfectant to prevent microbiological contamination are required in each animal facility. In animal facilities where many researchers and animal caretakers work, the property of being safe for human is very important for a control measure as well as its precision and efficacy. This study was conducted for the purpose of emphasizing the importance of control measures for viral contamination through the experience of detection of the origin of HVJ contamination and indicating the utility of the ELISA using purified LCMV-NP expressed by recombinant baculovirus as a safety serological test and WAHS as a safety disinfectant, both of which were very important control measures.

Ethics: All the animal experiments involved in this thesis were conducted in accordance with the NIID guidelines for animal experiments being in force at the time when each animal

experiment was performed.

Chapter 1

Detection of the Antibody to Lymphocytic Choriomeningitis Virus in Sera of Laboratory Rodents Infected with Viruses of Laboratory and Newly Isolated Strains by ELISA Using Purified Recombinant Nucleoprotein

ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed to detect the antibody against lymphocytic choriomeningitis virus (LCMV) in sera of laboratory animals. In this ELISA system, LCMV-nucleoprotein (NP) expressed by recombinant baculovirus and purified with high molar urea was used as the antigen. Sera from laboratory animals experimentally infected with the Armstrong strain or the newly isolated M1 strain of LCMV were examined to detect anti-LCMV antibody by the ELISA system, and the reactivity was compared with that of IFA test. Regardless of LCMV strain, all the sera of adult mice infected with LCMV were positive with very high optical density (OD). Also, the sera from mice neonatally infected with LCMV M1 strain were positive with slightly lower OD than adult mice. In contrast, all the sera of uninfected mice were negative to LCMV-NP antigen. Similarly, anti-LCMV antibodies were detected in all the sera of hamsters, mastomyses, and gerbils infected with the LCMV Armstrong strain. The results of the ELISA were in complete agreement with those of IFA, and indicated the high sensitivity and specificity of the ELISA system in the detection of anti-LCMV antibody. Because this ELISA system does not require handling infectious LCMV in the course of the antigen preparation and serological assay, there is no risk of contamination both to human in the laboratory and to animals in nearby animal facility. In addition, by using negative control antigen in parallel

with positive antigen in ELISA, the LCMV contamination in laboratory animals can be exactly checked.

INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV) is an RNA arenavirus and an important zoonotic pathogen. Although humans infected with LCMV commonly have no symptoms or experience only mild febrile illnesses, patients suffer aseptic meningitis in severe cases. House mice (*Mus musculus*) are the natural hosts of LCMV and transmit the virus to same and/or other species including humans (National Research Council, 1991b). Hamsters are also known to be susceptible to LCMV and are a potential source of prevalence of LCMV (Amman, *et al*., 2007, Parker *et al*., 1976). When LCMV infects immunocompetent adult mice, the virus is eliminated by the host immune response. However, if mice are infected with LCMV in utero or in the neonatal period, the virus infects persistently without marked clinical signs until immune complex glomerulonephritis occurs at 7-10 months of age (Hotchin and Collins, 1964). In such mice, infectious virus is permanently excreted in the urine, saliva, and milk (National Research Council, 1991b). From investigations of antibody against LCMV, wild house mice are commonly contaminated with LCMV on a global basis (Childs *et al*., 1992, Lledó *et al*., 2003, Morita *et al*., 1996). These mice become natural reservoirs and could transmit LCMV to laboratory animals (Knust, *et al*., 2014, Smith *et al*., 1984). Other than wild mice, the main source of LCMV infection in laboratory animals is transplantation of tumors contaminated with LCMV. Several cases have been reported in

which laboratory workers were accidentally infected with LCMV by contact with laboratory mice and Syrian hamsters implanted with tumors infected with LCMV (Biggar *et al*., 1977, Dykewicz *et al*., 1992, Hinman *et al*., 1975). Outside the laboratory, LCMV outbreaks have occurred among humans associated with pet hamsters (Biggar *et al*., 1975) and employees of feeder-rodent breeding facilities (Knust, *et al*., 2014). In recent years, LCMV infections in transplant recipients of organs from donor who had had contact with hamsters were reported in USA and France (Amman *et al*., 2007, Brouqui *et al*., 1995, Fischer *et al*., 2006).

In Japan, LCMV was first isolated from laboratory mice and guinea pigs in 1937 (Kasahara *et al*., 1937). Sato and Miyata (1986) reported that the antibody against LCMV was detected in laboratory animals in a survey of animal facilities in 1986. It has been the only report to suggest the possibility of LCMV infection in laboratory animals in Japan for decades after the first isolation of LCMV. Recently, in 2005, LCMV contamination was found in a wild-derived mouse strain imported from the Institut Pasteur (Paris, France) to RIKEN BioResource Center (Tsukuba, Japan) (Ike *et al*., 2007). This case reminds me that LCMV contamination should be monitored even though the contamination rarely occurs in Japan.

Since LCMV is a zoonotic pathogen, it is better not to use live LCMV in the monitoring system. Using a recombinant expressing system can reduce the risk of treating infectious

virus. In this study, I confirmed that the enzyme-linked immunosorbent assay (ELISA) system using recombinant LCMV-nucleoprotein (NP) antigen worked well for detecting anti-LCMV antibody. Using this system, I determined whether antibody from mice experimentally infected with a recently isolated LCMV strain cross-reacted to the recombinant antigen made from a well-documented laboratory strain of LCMV, since there is the possibility of antigenic variation among LCMV strains. I also determined antibody reactivity of sera from mice experimentally infected in the neonatal period, because it was reported that mice become tolerant to LCMV when they are infected in this period. The results show that this ELISA system was sensitive enough to detect the newly isolated strain including neonatal infection.

As well as mouse species, other rodents such as hamsters, gerbils (*Meriones unguiculatus*), mastomyses (*Praomys coucha*), etc., are used as laboratory animals. Although hamsters are well known to have the potential of being a LCMV reservoir, as described above, the susceptibility of gerbils and mastomyses to LCMV has not previously been investigated. Considering the wide range of hosts of LCMV, the risk of LCMV contamination from gerbils and mastomyses cannot be denied. In this study, I tried to detect anti-LCMV antibody in laboratory rodents including gerbils and mastomyses.

MATERIALS AND METHODS

Animals

Specific-pathogen-free (SPF) mice, gerbils, mastomyses, and hamsters were used for experimental infection with LCMV. Female C57BL/6CrSlc (6 weeks of age), C3H/HeSlc (6 weeks and pregnant), and Slc:ICR (4 weeks) mice were obtained from Japan SLC Inc. (Hamamatsu, Japan). The ICR mice were maintained for several months and used as sentinel animals in routine microbiological monitoring in the National Institute of Infectious Diseases Japan (NIID) facility. The sera from ICR mice were used as negative control samples. Female gerbils (MON/JmsGbsSlc) (6 weeks) were obtained from Japan SLC Inc.; male mastomyses (MCC) (4 weeks) and male hamsters (HAW) (12 weeks) maintained in the NIID facility were used for experimental infection. Sera used for negative control were obtained from the following animals maintained in the NIID facility: a female gerbil (Mg-W) (24 weeks), female gerbils (Mg-B) (24 and 48weeks), male and female mastomyses (MCC) (20 weeks), and female hamsters (HAW) (14, 16, and 38 weeks).

All the animals were nursed under barrier conditions and provided with water and commercial laboratory mouse chow *ad libitum*. Animal experiments were peer-reviewed by the Animal Care and Use Committee of the NIID and approved by the director of the NIID in accordance with the guides for animal experiments performed at the NIID.

Viruses

Recombinant baculovirus, which was inserted the coding region of the gene for the NP of LCMV WE strain using the transfer vector pAcYM1 (Matsuura *et al*., 1987), and polyhedrin-deleted baculovirus, which was constructed by co-transfection of baculovirus DNA and pAcYM1 with no coding region of the gene, were kindly provided by Dr. Matsuura (Research Institute for Microbial Diseases, Osaka University). Baculoviruses were propagated in Tn5 cells and the virus titers were determined by the 50% tissue culture infectious dose assay. Briefly, Tn5 cells were seeded into a 96-well tissue culture plate at a density of 8×10^3 per well and allowed to settle for 30 min at room temperature. Then, 50 μl of 1:10 serially diluted (10^{-1} to 10^{-7}) baculovirus stock was added to each well. Plates were incubated at 27℃ for 4 days and cell morphology was observed under an inverted microscope. The virus titers were calculated using the method described by Reed and Muench (1938).

The Armstrong strain and the M1 strain of LCMV were used for experimental infection. The M1 strain was isolated from wild-derived MAI/Pas mice persistently infected with LCMV which were imported from France and bred at RIKEN BioResource Center (Japan) (Ike *et al*., 2007). Both strains of LCMV were propagated in Vero E6 cells and virus titers were measured by counting the number of infected cell foci detected by the

peroxidase-anti-peroxidase method (PAP method) as previously described (Tanishita *et al*., 1984).

Preparation of LCMV-NP antigen

LCMV-NP antigen was prepared from Tn5 cells infected with the recombinant baculovirus expressing LCMV-NP at a multiplicity of infection (MOI) of 0.2. At 3 days post-infection, the cells were harvested and washed 3 times with PBS. The cells were resuspended in 1% NP40/PBS, allowed to stand on ice for 15 min, and centrifuged at 10,000 rpm for 10 min. The pellet was serially treated with urea solutions at different concentrations. First, the pellet was suspended in 1 M urea in 1% NP40/PBS, sonicated, and centrifuged at 8,000 rpm for 5 min. Then, the pellet was washed in PBS and suspended in 2 M urea in PBS. After the suspension was sonicated and centrifuged, the pellet was washed in PBS and suspended in 8 M urea in PBS. The suspension was sonicated and centrifuged, and the supernatant was used as LCMV-NP antigen. Negative control antigen was prepared likewise from Tn5 cells infected with polyhedrin-deleted baculovirus. To prepare crude LCMV-NP antigen, the infected Tn5 cells were resuspended in PBS and sonicated on ice 3 times for 20 sec according to the method of Homberger *et al*. (1995). The resulting cell extract was used as crude LCMV-NP antigen. Crude negative control antigen was prepared

likewise from Tn5 cells infected with polyhedrin-deleted baculovirus. The protein concentration of antigens was determined by using a commercial kit, Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). To assess the quality of purified antigens, an aliquot of antigens was electrophoresed on 10% SDS-PAGE gel and stained with 0.1% Coomassie Brilliant Blue R-250 in 30% methanol and 10% acetic acid.

LCMV infection

C57BL/6 mice and mastomyses were infected with 1×10^5 focus forming units (FFU) of the Armstrong strain of LCMV in PBS, intraperitoneally. Similarly, gerbils and hamsters were infected with 1.7×10^5 FFU and 2.5×10^5 FFU of the Armstrong strain of LCMV, respectively. At 4 weeks post infection, the animals were anesthetized with isoflurane, and sera were collected and kept at -80℃ until use for antibody detection.

C3H/He mice were used to produce persistent LCMV carrier mice because neonates of C3H genetic background mice are highly susceptible to LCMV (Doyle *et al*., 1980), and frequently used to produce LCMV carrier mice (Thomsen *et al*., 1985, Volkert *et al*., 1975). Neonatal C3H/He mice obtained from pregnant mice were infected with 6.9×10^3 FFU of M1 strain of LCMV, intraperitoneally, within 18 h after birth. Adult C3H/He mice were also infected with 6.9×10^4 FFU of M1 strain of LCMV, intraperitoneally. The animals

neonatally infected with LCMV at 6 or 8 weeks post infection and adult mice at 4 weeks post infection were anesthetized with isoflurane, and sera were collected and kept at -80°C.

Serological assays

Enzyme-linked immunosorbent assays (ELISA) were carried out using the LCMV-NP and negative control antigens prepared as above. The antigens were diluted 1:800 in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and 100 μl was added to each well of a 96-well NUNC-immuno plate with a polysoap surface (Nunc, Roskilde, Denmark). After incubation at 4ºC overnight, the plate was washed 3 times with 0.05% Tween 20 in PBS (Tween-PBS). Then, the plate was blocked with 10% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) in H_2O (blocking buffer) for 1 h at room temperature and washed 3 times with Tween-PBS. In ELISA with crude LCMV-NP and negative control antigens, 3% gelatin in PBS was used for blocking (Homberger *et al*., 1995). One hundred microliters of sera diluted 1:100 in blocking buffer were added to LCMV-NP antigen and negative control antigen wells, incubated for 1 h at room temperature, and washed 3 times with Tween-PBS. Peroxidase-labeled goat anti-mouse IgG (Zymed Laboratories, San Francisco, CA), goat anti-hamster IgG (KPL, Inc., Gaithersburg, MD), or goat anti-rat IgG (Zymed) was diluted 1:4,000, 1:12,000, or 1:4,000, respectively, in blocking buffer, and 100 μl was added to each

well. Since peroxidase-labeled anti-mastomys IgG and anti-gerbil IgG were not commercially available, both anti-mouse IgG and anti-rat IgG were alternatively used for the mastomys and gerbil systems. After the plate was incubated for 1 h at room temperature and washed 3 times with Tween-PBS, 100 μl of substrate solution consisting of 10 mg of o-phenylenediamine and 10 μl of H_2O_2 in 25 ml of 0.05 M phosphate-citrate buffer was added to each well and reacted in the dark for 30 min at room temperature. The reaction was stopped by adding 50 μl of 1 M H_2SO_4 and the chromogen produced was measured for absorbance at 492 nm.

To prepare the antigen for the immunofluorescence assay (IFA), HeLa 229 cells were infected with the Armstrong strain of LCMV and incubated at 37°C in a 5% CO_2 atmosphere. After cytopathic effect was observed, the cells were harvested and mixed with equal numbers of uninfected HeLa 229 cells. Then, about 1.3×10^4 cells in 10 μl PBS were spotted within printed circles of Teflon-coated glass slides (AR Brown, Tokyo, Japan) and air-dried with UV irradiation to inactivate LCMV for 2 h. After being washed with PBS, the cells were fixed with acetone for 10 min. The slides were stored at -80°C until use. Mouse sera diluted 1:100 or other animal species sera diluted 1:40 in PBS were applied to the IFA slides and incubated for 30 min at room temperature. Fluorescein isothiocyanate (FITC) -labeled anti-animal species IgGs were used as secondary antibodies. Rabbit anti-mouse IgG

(Zymed) was used for the mouse, mastomys, and gerbil systems, and goat anti-hamster IgG (KPL) was used for the hamster system. The slides were reacted with the secondary antibodies for 30 min at room temperature and then examined under a fluorescence microscope.

RESULTS

I developed an ELISA system using recombinant LCMV-NP antigen purified with sequential urea treatment. The protein concentration was 0.7 mg/ml in purified LCMV-NP antigen, 0.6 mg/ml in purified negative control antigen, and 4.7 mg/ml in both crude LCMV-NP and negative control antigen. Purified LCMV-NP and negative control antigens were analyzed by SDS-PAGE. As shown in Fig. 1-1, LCMV-NP antigen was detected as a protein band at 62 kDa with 2 minor low molecular weight bands, which were also detected in negative control antigen. To confirm the usefulness of the ELISA system with purified antigen, anti-LCMV-NP antibody in the sera of four species of animals experimentally infected with LCMV was examined by the ELISA. In addition, the detection rate of anti-LCMV antibody of ELISA with purified antigen was compared to that of the IFA test, which is a well-established method for anti-LCMV antibody detection.

To determine the reactivity of mouse sera, adult C57BL/6 mice were infected with a laboratory strain (Armstrong) and adult or neonatal C3H/He mice were infected with the newly isolated M1 strain of LCMV. Table 1-1 shows the anti-LCMV antibody detectability in the sera of mice in the ELISA test using recombinant LCMV-NP antigen or negative control antigen. In the mouse system, ELISA with crude LCMV-NP or negative control antigen was also performed and the results were compared with those of ELISA with purified

LCMV-NP or negative control antigen. In ELISA with purified antigens, regardless of the virus strains used, all the adult C57BL/6 and C3H/He mice infected with LCMV produced strong anti-LCMV antibody in the sera. Furthermore, C3H/He mice neonatally infected with LCMV M1 also showed strong anti-LCMV antibody response in sera with slightly lower optical density (OD) than adult mice. In ELISA with negative control antigen, 1 of 8 adult C57BL/6 mice infected with the Armstrong strain, 1 of 8 C3H/He adult mice infected with the M1 strain, and 1 of 9 C3H/He mice infected neonatally with the M1 strain showed comparatively high OD, over 0.4. Since the OD values of these sera to LCMV-NP antigen were 5 times more than those to negative control antigen, it was clear that the responses were specific to the LCMV-NP antigen. On the other hand, 20 sera from ICR adult mice that were uninfected were LCMV-negative with low ELISA OD. In ELISA with crude LCMV-NP antigen, in all the mice infected with LCMV, anti-LCMV antibody was detected with OD similar to that in ELISA with purified antigen. However, in ELISA with crude negative control antigen, all the averages of OD were over 0.4 and 15 of 25 mice examined showed high OD, over 0.5.

The anti-LCMV antibody detectability of other animal species is shown in Table 1-2. In the hamster system, anti-LCMV antibody was detected in all the sera of hamsters infected with the Armstrong strain of LCMV. Also in the sera of mastomys and gerbil, anti-LCMV

antibody was detected using both anti-mouse IgG and anti-rat IgG secondary antibodies. In particular, anti-rat IgG secondary antibody showed a very strong reaction in the gerbil system. All animals showed very low OD to negative control antigen in the hamster, mastomys, and gerbil systems. In addition, uninfected sera from 3 hamsters, 4 mastomyses, and 3 gerbils were LCMV-negative with low ELISA OD.

In IFA tests, anti-LCMV antibody was clearly detected in all the sera obtained from the adult animals infected with the Armstrong strain of LCMV (Fig. 1-2 A-D). Adult C3H/He mice infected with the M1 strain of LCMV also produced strong anti-LCMV antibody (Fig. 1-2 E). In contrast, 20 sera of negative control mice were LCMV-negative in the IFA test (Fig. 1-2 F).

Table 1-3 summarizes all the data described above. All the sera of animals infected with LCMV had LCMV-positive results in both ELISA and IFA tests, and the results of ELISA were in complete agreement with those of IFA (Table 1-3).

DISCUSSION

In the routine monitoring of laboratory animals, IFA and ELISA tests are usually used to detect anti-LCMV antibody (Hayashimoto *et al*., 2013, Sato and Miyata, 1986, Schoondermark-van *et al*., 2006, Smith *et al*., 1984). For the IFA test, cells infected with LCMV are spotted onto glass slides as antigen (Lewis *et al*., 1975, Sato and Miyata, 1986). Also, it has been reported the antigen is prepared from cells infected with LCMV for the ELISA test (Turkovic and Ljubicic, 1992). Since these antigens are inactivated by acetone fixation, UV irradiation, or gamma irradiation before use for the test, there is no risk of further contamination in the laboratory or nearby animal facility. However, during the preparation of the antigen, handling of infectious LCMV is required, and it is necessary to treat LCMV in a biosafety containment facility. On the other hand, handling of recombinant LCMV-NP as an antigen, as reported in this paper, does not require the use of infectious LCMV in the course of the antigen preparation and serological assay. Taken together, recombinant LCMV-NP is more convenient and safer than the inactivated antigen prepared from cells infected with LCMV.

ELISA with recombinant LCMV-NP has previously been reported by Homberger *et al*. (1995). In their paper, insect cells infected with recombinant baculovirus were harvested and sonicated in PBS, and the resulting cell extract was used as an antigen without further

purification. Their antigen preparation method was very simple and the ELISA system was almost as sensitive as IFA. However, some sera of mice infected with LCMV, which were positive in IFA, were negative in ELISA. In my results using antigens prepared by the same methods as Homberger *et al*. reported, all the sera from mice infected with LCMV showed strong reactions in ELISA. However, there was also a comparatively strong reaction in ELISA with crude negative control antigen as shown in Table 1-1. These findings suggest that LCMV-positive serum might be judged to be LCMV-negative by screening using the ELISA system with crude antigens because of a high background level.

Recently, it was reported that LCMV contamination was not found by ELISA using baculovirus recombinant LCMV-NP when the contamination occurred in Japan (Ike *et al*., 2007). In my experience, antigen from baculovirus infected cells is not easily extracted. Therefore, in the present study, I used LCMV-NP obtained by purification with high molar urea as the antigen and this antigen preparation seems to be more sensitive to anti-LCMV antibody than baculovirus recombinant LCMV-NP preparation without purification. In the detection of anti-LCMV antibody in the sera of animals experimentally infected with LCMV, all the sera were positive in both the IFA and ELISA tests and showed very high OD values in the ELISA test. On the other hand, most LCMV-positive sera showed very low OD values in ELISA with negative control antigen. In addition, all the sera of uninfected mice were

negative in both the IFA and ELISA tests and showed very low OD values in the ELISA test. These findings indicate the high sensitivity and specificity of the ELISA system using purified antigen in the detection of anti-LCMV antibody.

A possibility still existed that the newly isolated M1 strain was not cross-reactive for recombinant LCMV-NP made from the laboratory strain. In my results, the antigen made from WE strain reacted well with sera infected with the newly isolated virus, showing this ELISA system is effective among LCMV strains. The usability of this system to detect antibody in natural infection should be examined in a further study.

It is well known that anti-LCMV antibody in serum of mice persistently infected with LCMV is difficult to detect because of their tolerance to LCMV. However, Buchmeier and Oldstone (1978) showed the presence of antibodies against all LCMV structural peptides, even in mice neonatally infected with LCMV, and other investigators reported that anti-LCMV antibody was detected in the sera by double-sandwich ELISA with suspension of LCMV-carrier spleens as antigen (Thomsen *et al*., 1985). In fact, in the present study, the purified recombinant antigen was able to detect anti-LCMV antibody in all the sera of mice neonatally infected with the M1 strain. Further investigation, examining sera of mice infected in utero with LCMV, is necessary to confirm whether this recombinant LCMV-NP antigen is useful for the detection of anti-LCMV antibody in mice infected with LCMV

through vertical transmission.

The anti-LCMV antibody was detected in the sera from gerbils and mastomyses, suggesting that these animals are sensitive to LCMV. Although it is unknown whether or not these animals have the potential to be a reservoir of LCMV, it is desirable that these animals are periodically examined for LCMV contamination as well as mice and hamsters.

To exclude non-specific reactions of sample sera, it is useful that sera are reacted with negative control antigen in parallel with positive antigen in ELISA. In this study, Tn5 cells infected with polyhedrin-deleted baculovirus were used for the preparation of negative control antigen to exclude the reaction of serum to the materials derived from not only insect cells but also baculovirus. If an animal serum shows high antibody titer to LCMV-NP antigen, the reaction should be judged to be specific or non-specific to LCMV-NP antigen by comparison with the result of ELISA using the negative control antigen. Consequently, the precise LCMV specific antibody response can be obtained and hence I can accurately evaluate the LCMV contamination. Furthermore, the availability of this ELISA system for several laboratory rodents as demonstrated in this study suggests that this ELISA system is also applicable to the survey of wild rodents and humans outside the laboratory.

Recently, many laboratory mice are being transferred from overseas for research use. Due to the increase of the use of these mice, the possibility of LCMV contamination will be

higher in the future. To prevent LCMV contamination in the facility, laboratory animals should be routinely examined by a sensitive and precise method to detect the antibody against LCMV in spite of the fact that LCMV has not been prevalent in Japan for many years.

Table 1-1. ELISA reactivity of sera from mice infected with LCMV

Mouse	LCMV strain infected[a)]	Weeks post infection	No. of mice	ELISA titer (OD) : Means±S.D.			
				Purified antigen		Crude antigen	
				LCMV-NP	Negative control	LCMV-NP	Negative control
C57BL/6 (adult)	Armstrong	4	8	3.20±0.12	0.20±0.16	2.45±0.18	0.44±0.18
C3H/He (adult)	M1	4	8	3.02±0.08	0.19±0.13	2.89±0.18	0.51±0.19
C3H/He (neonate)	M1	6	4	2.05±0.43	0.12±0.04	2.48±0.19	0.69±0.29
C3H/He (neonate)	M1	8	5	2.45±0.44	0.18±0.14	2.84±0.17	0.92±0.53
ICR (adult)	Uninfected		20	0.08±0.06	0.04±0.04	NT[b)]	NT

[a)] Intraperitoneal inoculation.
[b)] NT, Not tested.

Table 1-2. ELISA reactivity of sera from hamsters, mastomyses, and gerbils infected with LCMV

Animals[a)]	LCMV strain infected[a)]	No. of animals	Second antibody	ELISA titer (OD) : Means±S.D.	
				LCMV-NP antigen	Negative control antigen
Hamster	Armstrong	3	Anti-Hamster	1.98±0.05	0.17±0.06
Hamster	—[b)]	3	Anti-Hamster	0.21±0.09	0.11±0.05
Gerbil	Armstrong	2	Anti-Mouse	1.11±0.46	0.03±0.01
			Anti-Rat	2.60±0.23	0.13±0.05
Gerbil	—	3	Anti-Rat	0.21±0.05	0.12±0.02
Mastomys	Armstrong	4	Anti-Mouse	1.68±0.19	0.03±0.01
			Anti-Rat	1.87±0.31	0.08±0.03
Mastomys	—	4	Anti-Rat	0.12±0.03	0.13±0.05

a) Four weeks post intraperitoneal inoculation.
b) Uninfected.

Table 1-3. Comparison of ELISA and IFA results in examinations of sera from mice, hamsters, mastomyses, and gerbils

Anti-LCMV Ab		Mouse				Hamster		Mastomys		Gerbil	
		Adult			Neonate						
IFA	ELISA	Arm[a)]	M1	Uninfected	M1	Arm	Uninfected	Arm	Uninfected	Arm	Uninfected
Positive	Positive[b)]	8	8	0	9	3	0	4	0	2	0
Positive	Negative	0	0	0	0	0	0	0	0	0	0
Negative	Positive	0	0	0	0	0	0	0	0	0	0
Negative	Negative	0	0	20	0	0	3	0	4	0	4

[a)] Strain of LCMV, intraperitonael inoculation. Arm means Armstrong strain.
[b)] Value obtained by subtracting OD of negative control antigen from OD of LCMV-NP, higher than 0.5 is positive.

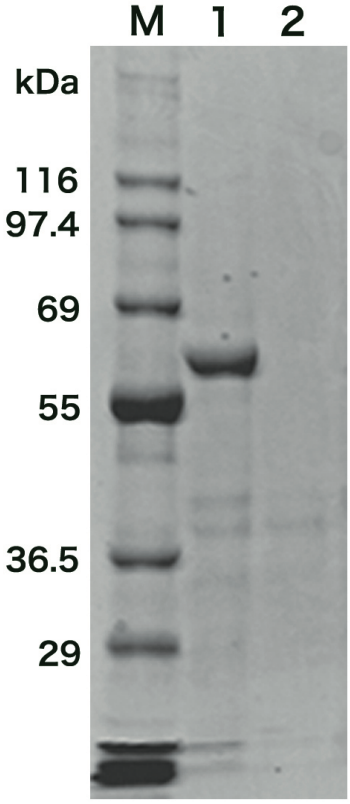

Fig. 1-1. SDS-PAGE analysis of purified recombinant proteins. Lane M, molecular weight marker proteins with the sizes indicated; lane 1, purified LCMV-NP antigen; lane 2, purified negative control antigen. A Protein band of LCMV-NP antigen, approximately 62 kDa (lane 1), was detected.

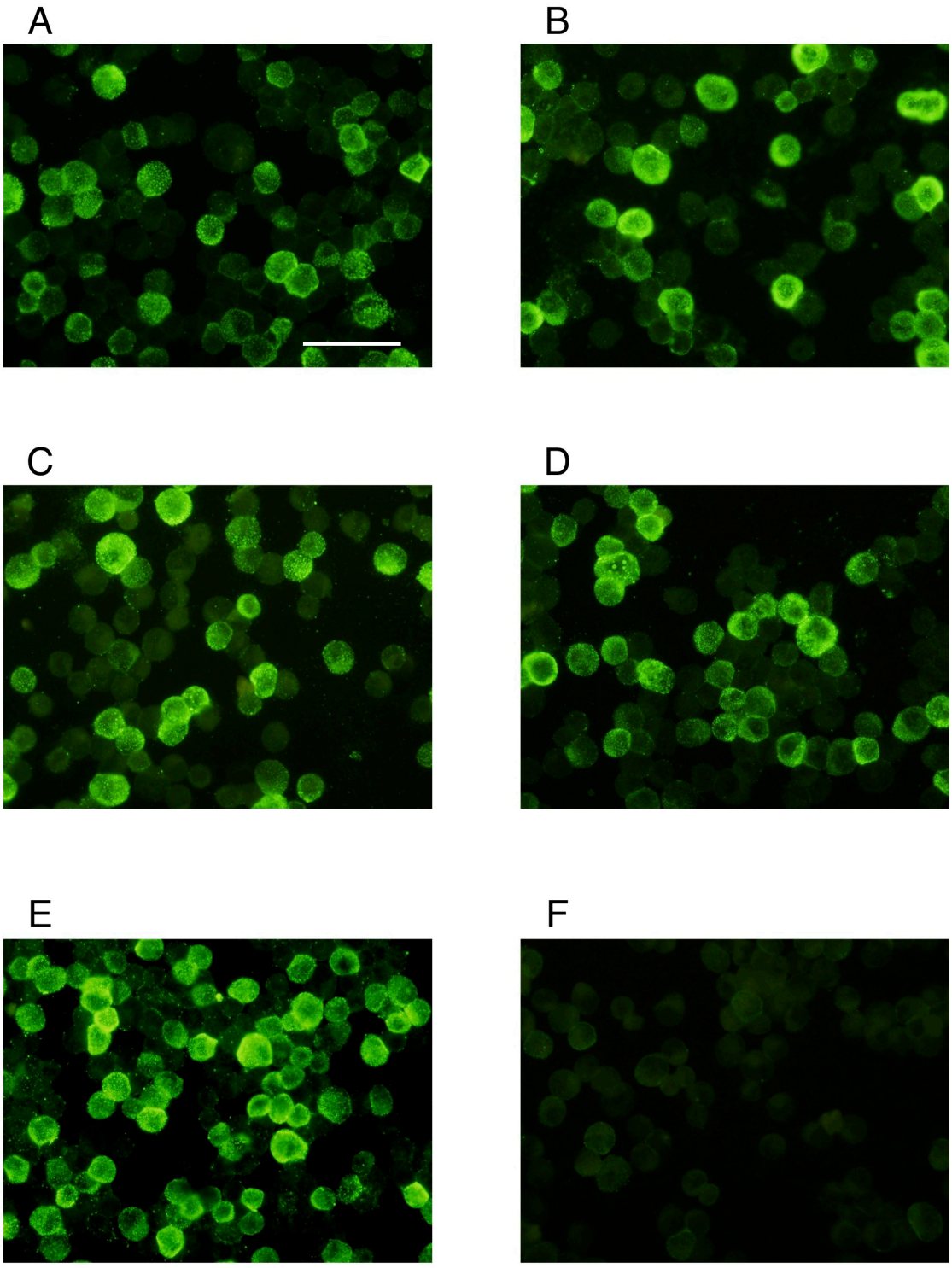

Fig. 1-2. IFA tests to detect the anti-LCMV antibodies in sera of each animal species. IFA slides were prepared using HeLa 229 cells infected with the Armstrong strain of LCMV. Scale bar = 50 μm. A to D represent the results of the Armstrong strain infected animal sera: A, mouse; B, hamster; C, mastomys; and D, gerbil. E represents the result of sera of adult mice at 4 weeks post infection with LCMV M1 strain. F represents the result of serum of negative control mouse.

Chapter 2

Contamination of Mouse-adapted Influenza Virus with Sendai Virus

ABSTRACT

In the laboratory animal facility of the National Institute of Infectious Diseases Japan (NIID), Sendai virus (HVJ) contamination occurred in a negative flow rack used for experimental infection with 4 strains of mouse-adapted influenza virus. Anti-HVJ antibody (Ab) was detected in 35/42 mice in the rack. It was considered that inoculated influenza virus stocks have been contaminated with HVJ. To specify the stock of influenza virus contaminated with HVJ, experimental infection was performed by using these strains in each vinyl isolator in order to avoid cross contamination between stocks. Anti-HVJ Ab was detected in all mice infected with a strain, A/Yamagata, at day 28 post-infection. In order to eliminate contaminated HVJ, this strain of influenza virus was passaged several times in cell cultures with anti-HVJ mouse serum. After these treatments, the virus stock was experimentally infected to mice and the results showed that infectious HVJ did not remain. Therefore, this virus strain was allowed for further experimental use in the NIID facility. Since then, HVJ contamination has not occurred in the NIID facility.

INTRODUCTION

Sendai virus (HVJ : hemagglutinating virus of Japan) is an enveloped negative-stranded RNA virus, also known as mouse parainfluenza virus type I. HVJ causes severe respiratory disease in mice and rats (Nakagawa *et al*., 1984). In laboratory animal facilities, HVJ is one of the most important pathogens to be prevented, because it is highly contagious and difficult to be controlled. Fortunately, a stable supply of HVJ-free mice has been provided by breeders in recent years. In the animal facility of the National Institute of Infectious Diseases Japan (NIID), the microbiological quality of mice is assured by monthly tests using sentinel animals. Cultivation of pathogenic bacteria as well as detection of antibodies (Ab) to murine viruses and bacteria by serological tests, such as enzyme-linked immunosorbent assay (ELISA) and complement fixation (CF) test are commonly conducted. Whenever anti-HVJ Ab is detected by serological tests, it has been recommended that the contaminated animal room must be rapidly cleaned by sacrificing the mice in the room and spraying appropriate disinfectants. In addition, it is important to elucidate the origin of HVJ outbreak in order to prevent further contamination.

In 1995, mice used as uninfected controls for experimental infection with mouse-adapted influenza viruses suddenly died in a negative flow rack in the NIID facility. Serological tests of other mice in the same rack revealed that mice were infected with HVJ, since

anti-HVJ Ab was detected. To specify the origin of HVJ contamination, sera of mice maintained in other racks in the same room were examined by ELISA, but no anti-HVJ Ab was detected in sera of any of mice examined. A specific pathogen-free (SPF) environment was maintained at the facilities of breeders which provide mice to the NIID facility. Also, routine monitoring in other areas of the NIID facility showed no HVJ contamination by that time. Unfortunately, the origin of HVJ infection was not specified at this time. When the experiment started again, anti-HVJ Ab was detected in serum of a sentinel mouse for routine monitoring in the rack used for experimental infection with mouse-adapted influenza viruses. Influenza virus stocks used in the experiment were prepared by means of passaging in mouse lungs and 10-day-old fertile chicken eggs (Tamura *et al*., 1992). There is a possibility that HVJ contamination was caused by inoculated influenza virus stocks made in the lungs of mice contaminated with HVJ. I report here the results of my research on the origin of HVJ contamination and elimination of HVJ from the contaminated virus stock.

MATERIALS AND METHODS

Mice

Female, SPF BALB/cCrSlc (4 weeks of age) and C3H/HeSlc mice (4 weeks) were obtained from Japan SLC Inc. (Hamamatsu, Japan). All the animals were nursed under barrier conditions and provided with commercial laboratory mouse chow and water *ad libitum*.

Serum sampling in the first contamination

Uninfected control mice suddenly died during the experiment of influenza virus infection performed by a facility user. Six BALB/c mice in the same negative flow rack (rack A) were sacrificed and sera were collected to investigate the cause of the sudden death of mice. After HVJ contamination was revealed by serological test of these mice, sera of 25 mice in other racks (rack B to F) in the same room were collected to confirm whether these mice were infected with HVJ or not.

Experiment of influenza virus infection causing the second contamination

About 2 months later from the first contamination, one of sentinel mice for routine monitoring was revealed as HVJ positive by serological test. This mouse was housed in the

negative flow rack in which mice used in the experiment of the same researcher of the first contamination was housed. According to the protocol provided by the principal researcher of the experiment, 42 mice (37 of BALB/c and 5 of C3H strain) were divided into 10 groups (group I to X) and 4 strains of influenza virus, A/Puerto Rico/8/34 (PR8, H1N1), A/Yamagata/120/86 (A/Yamagata, H1N1), B/Ibaraki/2/85 (B/Ibaraki), and A/Guizhou/54/89 ×A/Puerto Rico/8/34 (A/Guizhou-X, H3N2) were inoculated to mice in a negative flow rack. BALB/c mice of group I to III were immunized intranasally by dropping 1 μl of PBS containing formalin-inactivated HA vaccine prepared from PR8 (group I, 8 mice), A/Yamagata (group II, 1 mice), or B/Ibaraki (group III, 2 mice). BALB/c mice of group IV to VII were infected intranasally with 1 μl of mouse-adapted influenza viruses of PR8 (group IV, 9 mice), A/Yamagata (group V, 9 mice), A/Guizhou-X (group VI, 2 mice), or B/Ibaraki (group VII, 4 mice). Three weeks after the immunization or the infection, the mice of group I to VII were infected intranasally with 2 μl of PR8 virus. The mice were autopsied at day 18 after the challenge infection of PR8 virus, and sera were collected and kept at -80℃. A C3H mouse was infected intranasally with PR8 virus to determine the lethal dose 50 (group VIII). The period from the PR8 infection to the autopsy of the mouse of group VIII was not informed by the researcher. Four C3H mice (group IX) and 2 BALB/c mice (group X) were used as negative control groups.

Examination of influenza virus stocks for contamination with HVJ

In order to ascertain whether influenza virus stock was an origin for contamination or not, 6 and 5 BALB/c mice were used for the infection of A/Yamagata and B/Ibaraki strain, respectively, and 5 BALB/c and 5 C3H mice were used for PR8 strain. Mice were infected intranasally with 4 μl of each influenza virus stock. Then, the A/Yamagata and B/Ibaraki virus infected mice were kept together with 5 uninfected BALB/c mice each as a sentinel in a separate cage in each vinyl isolator until autopsy. C3H mice infected with PR8 virus were also kept together with 2 uninfected C3H mice as a sentinel. At day 28 post-infection (PI), the mice infected with A/Yamagata or B/Ibaraki strain and their sentinel mice were anesthetized with chloroform* and then exsanguinated from the heart with a syringe. Sera were collected by centrifugation and subsequently used for Ab determination. Similarly, the mice infected with PR8 strain and their sentinel mice were autopsied at day 25 or 35 PI.

(*Use of chloroform for anesthetizing was not prohibited in the NIID at this time.)

Elimination of HVJ from influenza virus stock

In order to eliminate HVJ from influenza virus stock, the virus stock was repeatedly passaged in the presence of anti-HVJ Ab. Briefly, anti-HVJ mouse serum was added to the influenza virus stock contaminated with HVJ and the mixture was incubated at 34 °C for 60

min. The mixture was then inoculated into Madin-Darby canine kidney cells and incubated in Eagle's minimum essential medium supplemented with 5 μg/ml of trypsin at 34°C for 3 days. Finally the supernatant was collected by centrifugation and diluted 1:10 in 0.01 M phosphate-buffered saline (PBS) (pH 7.3). The influenza virus stock was passaged in this manner another 2 times. The resulting virus stock obtained after the third passage was then diluted 1:10 in 0.01 M PBS (pH 7.3). After incubation with anti-HVJ mouse serum at 34 °C for 60 min, 0.2 ml of the suspension was injected into an embryonated egg and incubated at 34 °C for 48 hrs to make new influenza virus stock.

Confirming the elimination of HVJ in new influenza stock

To confirm the elimination of HVJ from A/Yamagata strain of new virus stock, 5 BALB/c mice were infected intranasally with 4 μl of A/Yamagata strain of influenza virus, and 4 uninfected BALB/c mice were maintained as a sentinel in a separate cage. All the mice were autopsied at day 28 PI, and the sera were subjected to Ab determination.

Serological assays

Anti-HVJ Ab in mice sera was examined by solid-phase ELISA and CF test. ELISA was carried out by using a commercially available HVJ-antigen (DENKA SEIKEN Co., Ltd.,

Tokyo, Japan). The antigen was diluted at 1:400 in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and 100 μl was added to each well of a 96-well NUNC-immuno plate with a polysoap surface (Nunc, Roskilde, Denmark). After incubation at 4ºC overnight, ELISA was performed in the same manner to detect anti-LCMV antibody as described in Chapter 1, except for use of peroxidase-labeled and affinity-purified Fab' portion of goat anti-mouse IgG (H+L-chain) (MBL, Nagoya, Japan) diluted at 1:2,000 in blocking buffer as a secondary antibody.

CF test was carried out by using the same HVJ-antigen as above, guinea pig sera as a complement, and hemolysin-conjugated sheep erythrocytes (Japan Lyophilization Lab., Tokyo, Japan). Mouse sera inactivated at 60ºC for 20 min were diluted at 1:10, 1:20, and 1:40 in gelatin veronal buffer (GVB^{2+}) consisting of 145 mM NaCl, 3.1 mM barbital, 1.8 mM sodium barbital, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% gelatin and 50 μl was added to each well of 96 well V-shaped plate. Then, 25 μl of HVJ-antigen diluted 1:8 in GVB^{2+} was added to each well followed by addition of 50 μl of 1:70 diluted guinea pig sera ($2CH_{50}$/ml) in cold GVB^{2+}. After incubation at 4ºC overnight, 50 μl of hemolysin-conjugated sheep erythrocytes diluted to 5×10^8 cells/ml in GVB^{2+} after washing in GVB^{2+} by centrifugation at 1,500 rpm for 10 min was added to each well. After the plate was mixed well, incubated at 37ºC for 1 h, and placed at room temperature for 1 h, mouse serum indicating less than 25%

of hemolysis in the plate was judged as HVJ positive.

ELISA to detect anti-influenza virus Ab in mouse sera was performed as described previously (Tamura *et al*., 1990), with purified hemagglutinin (Phelan *et al*., 1980) and anti-mouse IgG (γ-chain specific) conjugated with alkaline phosphatase (Zymed Laboratories, Inc., San Francisco, CA, USA). The chromogen was produced with *p*-nitrophenyl-phosphate and measured for absorbance at 410 nm, and the IgG Ab concentration was calculated by use of the 64-units-standard for HA-specific IgG prepared as described previously (Tamura *et al*., 1990).

RESULTS

Uninfected control mice for experimental mouse-adapted influenza virus infection suddenly died in a negative flow rack in the NIID facility. Serological tests of other 6 BALB/c mice in the same rack (rack A) were performed to investigate the cause of the sudden death of mice. Anti-HVJ Ab was detected in sera of all the mice examined (Table 2-1), indicating that HVJ contamination occurred in the room. To specify the origin of HVJ contamination, sera of 25 mice maintained in other racks (rack B to F) in the same room were examined by ELISA, but no anti-HVJ Ab was detected in sera of any of mice examined (Table 2-1). All the mice reared in the room were sacrificed and the room was thoroughly disinfected using 70% ethanol and iodine-based disinfectant. Because mouse-adapted influenza virus used for the experiment was not supposed to be a candidate of the cause of HVJ contamination, the origin of HVJ contamination was not specified at this moment.

The second HVJ contamination was revealed by serological tests of a sentinel mouse in routine monitoring. In the same negative flow rack in which the HVJ seropositive sentinel mouse was maintained, experimental infection was performed using 42 mice and 4 strains of mouse-adapted influenza virus by the same researcher of the first contamination. Sera of all the mice used for the experiment in the rack were subjected to the determination of anti-HVJ Ab and anti-influenza virus Ab, because only the mice used for the experimental infection

with mouse-adapted influenza viruses were seropositive to HVJ when the first HVJ contamination occurred. Table 2-2 shows the rate of detection of anti-HVJ Ab and anti-influenza virus Ab in each experimental group. Anti-HVJ Ab was detected in the sera of mice in all groups by ELISA. All C3H mice had anti-HVJ Ab in group VIII and IX by both ELISA and CF test. Also, anti-HVJ Ab was detected in most of the BALB/c mice examined by ELISA.

However, it was unknown which strain was contaminated with HVJ. There is also the possibility that anti-influenza virus Ab cross-reacts with HVJ antigen. To clarify which strain was contaminated and whether HVJ and influenza virus were cross reactive, experimental infection was performed by using A/Yamagata and B/Ibaraki strains which showed a high CF titer of anti-HVJ Ab, and PR8 strain which was used for all experimental groups. 6 and 5 BALB/c mice were infected with A/Yamagata and B/Ibaraki strain, respectively, and 5 BALB/c and 5 C3H mice were infected with PR8 strain. Mice infected with each influenza strain, except for BALB/c mice with PR8, were kept together with each sentinel mice in a separate cage in each vinyl isolator until autopsy. Table 2-3 shows the detection rates and ELISA titers of anti-HVJ Ab and anti-influenza virus Ab in the sera from the mice. In the sera of mice infected with B/Ibaraki or PR8 strain, only anti-influenza virus Ab was detected. On the other hand, both anti-influenza virus Ab and anti-HVJ Ab were

detected in all the mice infected with A/Yamagata strain. In addition, anti-HVJ Ab was detected in all the sentinel mice, but anti-influenza virus Ab was detected in only 2 of 5 sentinel mice in the same isolator where mice infected with A/Yamagata strain were maintained. These results made it clear that A/Yamagata strain of influenza virus stock was contaminated with HVJ.

Since there was a need for using influenza virus stock in an animal experiment, I tried to eliminate HVJ from influenza virus stock. A/Yamagata strain of influenza virus stock was treated with anti-HVJ Ab to eliminate HVJ as described under MATERIALS AND METHODS. To confirm whether HVJ was certainly eliminated from A/Yamagata strain of influenza virus, experimental infection with A/Yamagata strain of influenza virus was carried out. Table 2-4 shows the detection rates and ELISA titers of anti-HVJ Ab and anti-influenza virus Ab in the sera from the mice. Anti-HVJ Ab was not detected in any mice, either infected or not infected with A/Yamagata strain. From this result, A/Yamagata strain of influenza virus stock was determined not to contain HVJ and allowed for further experimental use in the NIID facility. In addition, it was denied that anti-influenza virus Ab cross-reacted with HVJ antigen.

DISCUSSION

In the animal facility of the NIID, HVJ contamination occurred during an experiment of influenza virus infection in an animal room. HVJ is well known to be transmitted by respiratory aerosol and highly contagious. Therefore, I thought that almost all mice should be infected with HVJ in this HVJ-contaminated room. However, only the mice in the rack used for experimental infection with influenza virus were infected with HVJ, while all the mice housed in other racks in the same room were seronegative to HVJ. This finding was so helpful for me to think that the contamination of HVJ was related only to the experimental infection with influenza virus and that the HVJ contamination was limited in mice reared in the same negative flow rack. All the mice including HVJ-contaminated mice were housed in negative flow racks in this animal room. The facts that negative airflow could prevent aerosol containing HVJ from leaking out of the rack and minimize the extent of HVJ contamination reminded me of the efficacy of negative flow rack and the importance of airflow control in an animal facility.

Mouse-adapted influenza virus stock used in the experiment was a candidate of an origin of HVJ contamination. In order to elucidate the origin, influenza virus stocks used for the research were separately inoculated to mice and the results revealed that A/Yamagata strain of influenza virus was contaminated with HVJ. In addition, all the sentinel mice kept with

A/Yamagata virus infected mice were HVJ-positive, though only 2 of 5 sentinel mice were seropositive to A/Yamagata virus with low ELISA titer. These findings indicated that HVJ was transmitted easily to mice by respiratory aerosol and transmission of mouse-adapted influenza virus by respiratory aerosol was less efficient.

Similar to this HVJ contamination, there are many reports on contaminated biological materials with pathogens. For instance, it was reported that transplantable tumors or tumor cell lines were contaminated with murine pathogens, such as MHV, LCMV, minute virus of mice, lactic dehydrogenase elevating virus (LDV), reovirus type 3, polyoma virus, *Mycoplasma pulmonis*, and *Helicobacter hepaticus* (Bhatt *et al*., 1986, Chen and Plagemann, 1997, Collins and Parker, 1972, Fox *et al*., 1977, Goto *et al*., 2001, Mahy *et al*., 1991, Nakai *et al*., 2000, Nicklas *et al*., 1993, Parker *et al*., 1970, Takakura *et al*., 2000, Yoshimura *et al*., 1997). The murine pathogen contaminations of various cell lines including tumor cells, embryonic stem cells, hybridoma cells, and cancer cells were recently summarized in other article (Peterson, 2008). These murine pathogens in tumors have affected the health of animals and the results of experiments. Fox *et al*. (1977) reported that mice became suffered from unexpected illness and died during serial passage of myelomas, from which MHV was isolated by subsequent investigation. Also, early deaths of mice inoculated with a sarcoma contaminated with LCMV were observed (Bhatt *et al*., 1986). In the case of LCMV

contaminated tumor, the contamination also caused an outbreak of LCMV infection in human which was associated with exposure to nude mice carrying tumor cells contaminated with LCMV (Mahy *et al*., 1991). Even if murine pathogen transmitted by biological materials does not lead animals to illness or death, many pathogens have potential risk to alter the results of experimental responses of immunity by affecting the susceptibility to other pathogen (Carrano *et al*., 1984, Dempsey *et al*., 1986, Mims and Wainwright, 1968), cytokine gene expression (Colle *et al*., 1993), immune system reactivities (Borrow *et al*., 1995), and the activity of macrophage (Blanden and Mims, 1973), natural killer cell (Lai *et al*., 1987, Schindler *et al*., 1982), cytotoxic T-lymphocite (Thomsen *et al*., 1982, Wijburg *et al*., 1996), IFN-gamma (Schijns *et al*., 1996), monocyte procoagulant (Levy *et al*., 1981), and humoral antibody (Michaelides and Simms, 1980). In addition, many other influences of murine pathogens on animal experiments have been described (Baker 1998, Besselsen *et al*., 2008, Nicklas *et al*., 1999). Besides, these biological materials are introduced into multiple laboratories to use for experiment, resulting in spreading the contamination not only in Japan but also worldwide. Therefore, the materials derived from animals should be responsibly managed and subjected to a microbiological examination, such as mouse antibody production (MAP) test (Nicklas *et al*., 1993) and PCR test (Bootz *et al*., 2003), before subsequent experimental use.

An adaptation of human or animal influenza virus to mice has been generally performed to use for animal experiments using mice. Mouse-adapted influenza virus is important and useful to evaluate the efficacy of influenza virus vaccine (Fan *et al*., 2004, Govorkova and Smirnov, 1997, Tamura *et al*., 1992) and anti-influenza virus drugs (Nguyen *et al*., 2012, Smee *et al*., 2012), and to investigate the change in viral virulence, tissue tropism, pathogenicity, and amino acid sequence during adaptation of influenza virus to a new mammal host (Brown *et al*., 2001, Ilyushina *et al*., 2010, Narasaraju *et al*., 2009, Sakabe *et al*., 2009, Shinya *et al*., 2007, Wang *et al*., 2012). On the other hand, because influenza virus is adapted to mice by serial lung-to-lung passages in mice, there is a potential risk that influenza virus is contaminated with pathogen originating from a donor mouse. Indeed, Fukumi *et al*. have reported that HVJ was isolated during the passage of influenza virus through mice (Fukumi *et al*., 1954). Since A/Yamagata strain of influenza virus stock had been adapted to mice by serial lung-to-lung passages in another institute, it is supposed that mice used in making virus stock was contaminated with HVJ. According to a serological survey in mouse sera collected from research and breeding colonies during 1983 to 1985 in Japan, HVJ was not detected in barrier mouse colonies but was one of main contaminants in conventional mouse colonies (Kagiyama *et al*., 1986). Also, in a survey in 1996, HVJ was still prevalent in non-SPF laboratory mice in USA (Jacoby and Lindsey, 1998). Judged from these

microbiological statuses of laboratory mice, it is speculated that non-SPF mice infected with HVJ were used for the lung-to-lung passage of A/Yamagata strain of influenza virus, though the history of passage in mice of A/Yamagata strain of influenza virus was not clear.

In our research, it was necessary to prepare uncontaminated A/Yamagata strain of influenza virus. So, immediately after A/Yamagata strain of influenza virus was revealed to be contaminated with HVJ, the virus stock was treated with anti-HVJ Ab to eliminate HVJ. The treated A/Yamagata strain of influenza virus was confirmed to be free from HVJ by experimental infection using mice and fortunately allowed to be used for experiment in the NIID facility. This result suggested a possibility of rescuing other virus stocks contaminated with murine virus by *in vitro* treatment with Ab against the certain murine virus. Similarly, some researchers have reported the elimination of murine pathogen, such as LDV and MHV from contaminated transplantable tumors. LDV, the most common viral contaminant in transplantable tumors (Nicklas *et al*., 1993, Nakai *et al*., 2000), was eliminated by *in vitro* passage of contaminated tumors (Nakai *et al*., 2000). Contaminated tumors with MHV were passaged in nude rats and then became free of MHV (Takakura *et al*., 2000). Though contaminated biological materials should be discarded and renew to contaminant-free materials, these methods to eliminate contaminants may be useful for unavailable or rare transplantable tumors.

As long as passage through animals is performed, the potential risk of contamination with murine pathogen is still present. Recently, viral contamination in mice is hardly found in current well-controlled animal facilities in Japan (Hayashimoto *et al*., 2013). However, there are still many conventional animal facilities in Japan. Non-SPF mice are supposed to be used for animal experiments including production and passage a new biological material. Stored biological materials without passage history should be confirmed to be free of viral contaminants by suitable microbiological examinations before experimental use to avoid the contamination of murine viruses in animal facilities.

Table 2-1. Detection of anti-HVJ antibody in mice in each rack in the HVJ contaminated room

Rack No.	Mouse strain	No. of positive / No. of animals examined ELISA titer : Means±S.D.
A	BALB/c	6/6 2.18±0.36[a)]
B	BALB/c	0/9 0.03±0.01
C	C3H	0/7 0.16±0.04
D	BALB/c	0/5 0.08±0.02
E	ICR	0/2 0.09±0.05
F	ICR	0/2 0.04±0.01

[a)] Value lower than 0.5 is negative.

Table 2-2. Detection of anti-HVJ antibody and anti-influenza virus antibody in mice in the HVJ contaminated rack

Group					No. of positive / No. of animals examined				
	Primary				ELISA				CF
	Immunization[a)]	Infection[b)]	Challenge[c)]	Mouse strain	Anti-A/Yamagata	Anti-B/Ibaraki	Anti-PR8	Anti-HVJ	Anti-HVJ
I	PR8 vaccine		PR8	BALB/c	NT [d)]	NT	8/8	8/8	5/8
II	A/Yamagata vaccine		PR8	BALB/c	1/1	0/1	1/1	1/1	0/1
III	B/Ibaraki vaccine		PR8	BALB/c	NT	NT	0/2	2/2	1/2
IV		PR8	PR8	BALB/c	NT	NT	NT	4/9	3/9
V		A/Yamagata	PR8	BALB/c	9/9	NT	NT	9/9	9/9
VI		A/Guizhou-X	PR8	BALB/c	NT	1/2	NT	2/2	1/2
VII		B/Ibaraki	PR8	BALB/c	NT	4/4	NT	3/4	3/4
VIII		PR8		C3H	NT	NT	NT	1/1	1/1
IX		Non treatment		C3H	NT	0/4	NT	4/4	4/4
X		Non treatment		BALB/c	NT	0/1[e)]	NT	1/2	0/2

[a)] Mice were immunized intranasally with 1 μl of PBS containing each HA vaccine.
[b)] Mice were infected intranasally with 1 μl of each mouse-adapted influenza virus stock.
[c)] Three weeks after the immunization or the infection, mice were infected intranasally with 2 μl of PR8 strain of influenza virus. Eighteen days after the challenge, mice were autopsied.
[d)] NT, Not tested
[e)] One mouse in group **X** was examined.

Table 2-3. Detection of antibody in sera of mice infected with A/Yamagata, B/Ibaraki or PR8 strain of influenza virus

Influenza virus strain	Mouse strain	Group (DPI[a])	No. of positive / No. of animals examined ELISA titer : Means±S.D.			
			Anti-A/Yamagata (unit/ml)	Anti-B/Ibaraki (unit/ml)	Anti-PR8 (unit/ml)	Anti-HVJ (OD)
A/Yamagata	BALB/c	Infection (28)	6/6 1235±321[b]	NT[f]	NT	6/6 2.27±0.21[e]
		Sentinel (28)	2/5 273±255	NT	NT	5/5 2.06±0.14
B/Ibaraki	BALB/c	Infection (28)	0/5 93±48	5/5 2796±1130[c]	NT	0/5 0.33±0.03
		Sentinel (28)	0/5 79±45	1/5 90±62	NT	0/5 0.28±0.04
PR8	C3H	Infection (25)	NT	NT	2/2 1974±171[d]	0/2 0.11±0.01
		Infection (35)	NT	NT	3/3 1340±782	0/3 0.19±0.03
		Sentinel (35)	NT	NT	0/2 16±21	0/2 0.15±0.02
	BALB/c	Infection (25)	NT	NT	2/2 791±699	0/2 0.08±0.02
		Infection (35)	NT	NT	3/3 1361±702	0/3 0.11±0.01

[a] Days post infection.
[b] Value lower than 200 is negative.
[c] Value lower than 200 is negative.
[d] Value lower than 100 is negative.
[e] Value lower than 0.5 is negative.
[f] NT, Not tested

Table 2-4. Detection of antibody in sera of mice infected with A/Yamagata strain of influenza virus treated with anti-HVJ antibody

Group (28 DPI [a])	No. of positive / No. of animals examined ELISA titer : Means±S.D.	
	Anti-A/Yamagata(unit/ml)	Anti-HVJ(OD)
Infection	4/5 1532±884[b]	0/5 0.22±0.02[c]
Sentinel	0/4 0	0/4 0.22±0.02

[a] Days post infection.
[b] Value lower than 200 is negative.
[c] Value lower than 0.5 is negative.

Chapter 3

Effect of Hypochlorite-based Disinfectants on Inactivation of Murine Norovirus and Attempt to Eliminate or Prevent Infection in Mice by Addition to Drinking Water

ABSTRACT

I evaluated the *in vitro* efficacy of weak acid hypochlorous solution (WAHS) against murine norovirus (MNV) by plaque assay and compared the efficacy with diluted NaOCl (Purelox) and 70% ethanol. WAHS was as effective as 70% ethanol and diluted Purelox for 0.5-min reactions. For 0.5-min reactions in the presence of mouse feces emulsion, the efficacy of WAHS and 1:600 diluted Purelox was decreased, reducing the virus titers by 2.3 and 2.6 $\log_{10}$, respectively, while 70% ethanol reduced the titer by more than 5 $\log_{10}$. However, WAHS showed more than 5 $\log_{10}$ reductions for the 5-min reaction even in the presence of feces emulsion. Since WAHS showed enough efficacy in inactivating MNV *in vitro*, I tried to eliminate MNV from MNV-infected mice by substituting WAHS for their drinking water. However, MNV was found to be positive in feces of mice drinking WAHS by an RT-nested PCR and plaque assay. To investigate whether hypochlorite-based disinfectants could prevent infection of a mouse with MNV, WAHS or 1:6,000 diluted Purelox was substituted for the drinking water of mice for 2 or 4 weeks, and then the mice were placed in a cage with an MNV-infected mouse. The supply of disinfectants was continued after cohabitation, but MNV was detected in the feces of all the mice at 1 week after cohabitation. In this study, I tried to eliminate and prevent MNV infection from mice by supplying hypochlorite-based disinfectants as an easy and low-cost method.

Unfortunately, drinking disinfectants was ineffective, so it is important in the case of MNV infection to keep the facility environment clean by use of effective disinfectants. Also, animals introduced into facilities should be tested as MNV free by quarantine and periodically confirmed as MNV free by microbiological monitoring.

INTRODUCTION

Noroviruses are nonenveloped, positive-sense RNA viruses that belong to the genus *Norovirus* in the family *Caliciviridae* (Zheng *et al*., 2006). Human noroviruses cause acute nonbacterial gastroenteritis worldwide and are transmitted through the fecal-oral route, usually by eating food or drinking liquids contaminated with noroviruses (Patel *et al*., 2009). Noroviruses are divided into five major genogroups, that is, GI, which infects humans; GII, which infects humans and swine; GIII, which infects bovine; GIV, which infects humans and canines; and GV, which infects mice (Patel *et al*., 2009). Murine norovirus (MNV) was first discovered in 2003 in laboratory mice that were deficient in signal transducer and activator of transcription 1 (STAT1) and recombination-activating gene 2 (RAG2) (Karst *et al*., 2003). Surveillance of microbiological contamination of MNV shows that MNV is the most prevalent viral pathogen in laboratory animal facilities in the USA, Canada, and Australia (Hsu *et al*., 2005, McInnes *et al*., 2011). Also, in Japan, it was reported that MNV is one of the most prevalent pathogens in conventional mouse colonies (Goto *et al*., 2009, Kitagawa *et al*., 2010). The effect of MNV infection on research using mice has not been revealed completely. Some reports showed MNV did not affect the results of research (Goto *et al*., 2009, Hsu *et al*., 2006, Karst *et al*., 2003, Kitagawa *et al*., 2010, Müller *et al*., 2007). On the other hand, there are reports on clinical signs such as weight loss, gastric bloating, and

diarrhea in STAT1-deficient mice infected with MNV, and a modest inflammatory response and increase in necrotic cells in the small intestine of MNV-infected immunocompetent mice (Kahan *et al*., 2011). In research that strictly requires a normal immune system, it is better to prevent contamination of animal facilities with MNV and to eliminate MNV from mice contaminated with MNV.

While human norovirus is unable to grow *in vitro* (Duizer *et al*., 2004, Lay *et al*., 2010), MNV can replicate in both cultured cells and mice (Cox *et al*., 2009, Wobus *et al*., 2004). Therefore, MNV has been used to study proteolytic processing, environmental stability and inactivation (Bae and Schwab, 2008, Belliot *et al*., 2008, Cannon *et al*., 2006, Sosnovtsev *et al*., 2006, Wobus *et al*., 2006). Belliot *et al*. (2008) previously examined the efficacy of some disinfectants, and showed that ethanol, povidone-iodine, and sodium hypochlorite were effective against MNV. In animal facilities, these disinfectants have been used to keep the environment of facilities clean. Because of the high antiseptic efficacy, low cost, and safety for humans, weak acid hypochlorous solution (WAHS) is beginning to be used in animal facilities, hospitals, and food industries. WAHS is composed of sodium hypochlorite blended with hydrochloric acid in tap water, with the pH value adjusted to 6.0–6.4 and the residual chlorine concentration adjusted to about 60 ppm. The main effective form of chlorine in WAHS is hypochlorous acid (HOCl). The efficacy of WAHS against various

microorganisms has been reported (Ono *et al*., 2012, Taharaguchi *et al*., 2014). In this study, I first evaluated the virucidal effect of WAHS against MNV *in vitro*. The efficacies of several dilutions of sodium hypochlorite (NaOCl) and 70% ethanol in inactivating MNV were also tested for comparison with WAHS. Though the main effective form of chlorine in NaOCl is also HOCl as same form of WAHS, NaOCl corrodes metal due to its strong alkalinity and has disadvantages such as its irritant properties and strong odor (Fukuzaki, 2006). Compared with NaOCl, WAHS is a suitable substitute for drinking water of animals. I attempted to eliminate MNV from mice experimentally infected with MNV and to prevent mice from becoming infected with MNV by substituting WAHS for their drinking water and compared the results with those obtained with NaOCl.

MATERIALS AND METHODS

Mice

Female, specific-pathogen-free (SPF) Slc:ICR mice (6 weeks of age) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and used for experimental infection with MNV. The breeder's health monitoring report indicated that these mice were free of the following intestinal microorganisms: *Pseudomonas aeruginosa*, *Citrobacter rodentium*, *Salmonella* spp., *Corynebacterium kutscheri*, *Clostridium piliforme,* pinworm, intestinal protozoa, *Helicobacter hepaticus*, *Helicobacter bilis*, and MNV. They were also negative for mouse hepatitis virus, Sendai virus, ectromelia virus, lymphocytic choriomeningitis virus, pneumonia virus of mice, mouse adenovirus, *Mycoplasma pulmonis*, *Pasteurella pneumotropica*, Cilia-associated respiratory bacillus, ectoparasites, and Dermatophytes. All the animals were bred under barrier conditions and provided with commercial laboratory mouse chow and water *ad libitum* unless otherwise indicated. Animal experiments were peer-reviewed by the Animal Care and Use Committee of the NIID and approved by the director of the NIID in accordance with the guides for animal experiments performed at the NIID.

Cells and viruses

RAW264.7 cells were purchased from ATCC (Manassas, VA, USA) and maintained in high-glucose Dulbecco's modified essential medium (DMEM) (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), and 0.1 mg/ml kanamycin (DMEM-FBS). The S7 strain of MNV (MNV-S7), which was isolated from a conventional mouse in Japan, was used in this study. A monolayer of RAW264.7 cells grown in a tissue culture flask was infected with MNV-S7 at a multiplicity of infection (MOI) of 0.1 and incubated in DMEM-FBS for 3 h at 37°C in a 5% CO_2 atmosphere. After the cultured medium was removed, the cells were incubated in new DMEM-FBS for 2 days. Then, the cells were subjected to freezing-thawing, and the cultured medium was centrifuged at 3,000 rpm for 5 min at 4°C. The supernatant was used as the virus stock throughout the study.

Plaque assay

RAW264.7 cells were seeded into 12-well plates at a density of 8.5×10^5 viable cells per well. After the culture medium was removed, 0.3 ml of 1:10 serially diluted (10^{-1} to 10^{-6}) samples in DMEM-FBS were added to each well. Plates were incubated for 2 h at 37°C in a 5% CO_2, and the inocula were removed. After washing with DMEM, the cells were overlaid with 1 ml of 1.5% Agar Noble (Becton, Dickinson and Company, Sparks, MD, USA) in

Eagle's Minimum Essential Medium (EMEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% FBS, 2 mM L-glutamine, and 0.22% $NaHCO_3$ per well. Plates were incubated for 2 days at 37°C in 5% CO_2. To visualize plaques, the cells were overlaid with 1 ml of 1.5% Agar Noble in EMEM supplemented with 10% FBS, 2 mM L-glutamine, and 0.01% neutral red per well, followed by incubation for 4-5 h.

Evaluation of disinfectant efficacies against MNV *in vitro*

Three disinfectants were used to inactivate the MNV. Ethanol was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Purelox (6% NaOCl) was purchased from Oyalox (Tokyo, Japan). Weak acid hypochlorous solution (WAHS) was produced using a Steri Revo HSP-SR-600 (HSP Corp., Okayama, Japan), which blended sodium hypochlorite with hydrochloric acid and adjusted the pH value to 6.0–6.4 and the residual chlorine concentration to about 60 ppm. Ethanol was diluted in distilled water to a final concentration of 70%, and Purelox was diluted at 1:200 (over 200 ppm of residual chlorine concentration), 1:400 (150 to 200 ppm), 1:600 (100 ppm), 1:800 (70 ppm), and 1:1,000 (60 ppm) in distilled water. WAHS was used without dilution.

To investigate the influence of organic materials, fresh feces were collected from an ICR mouse that was maintained as a sentinel animal in routine microbiological monitoring in

the NIID facility and homogenized in 9 vol. (w/v) of PBS. After centrifugation at 3,000 rpm for 1 min, the supernatant was used as a feces emulsion.

Inactivation of MNV was performed by adding 0.9 ml of the disinfectant to 0.05 ml of virus stock solution (8.8×10^7 PFU/ml) and 0.05 ml of PBS. In the case of presence of the feces emulsion, the virus solution was mixed with 0.05 ml of mouse feces emulsion instead of PBS. Immediately after the disinfectant was added to the virus solution, the reaction mixtures were vortexed and incubated for 0.5, 1, or 5 min at room temperature. As inactivation control samples, virus solution was incubated for 5 min with PBS instead of disinfectant. To stop the reaction, 9 ml of DMEM-FBS was added to the reaction mixture. Then, each solution was serially diluted 1:10 in DMEM-FBS, and the virus titer was measured by the plaque assay as described above.

Attempt to eliminate MNV from infected mice by substituting WAHS for drinking water

Six ICR mice divided into 2 groups were used. Mice were orally infected with 1×10^6 plaque forming units (PFU) of MNV in PBS. At 1 week post infection, the drinking water for 3 mice was changed to WAHS and then repeatedly exchanged with fresh WAHS every other day. At 1, 2, 3, and 4 weeks post infection, feces were collected from each mouse.

At 5 weeks post infection, mice were sacrificed, and the duodenum, jejunum, cecum, rectum, and feces were collected and kept at -80°C until use. The presence of viable virus in each intestine and feces was investigated by RT-nested PCR, and the virus titer was determined by plaque assay.

Attempt to prevent MNV infection by substituting hypochlorite-based disinfectants for drinking water

The drinking water of mice was changed to WAHS or 1:6,000 diluted Purelox and repeatedly exchanged with fresh disinfectant every 3 or 4 days during the experiment. After 2 or 4 weeks of supplying the disinfectant in place of water, 3 mice were moved into the same cage as a mouse that had been orally infected with 1×10^6 PFU of MNV 1 week before cohabitation. At 1, 2, and 3 weeks post cohabitation, feces were collected from each mouse. The presence of viable virus in feces was investigated by RT-nested PCR.

Extraction of RNA and RT-nested PCR of mouse samples

Feces were homogenized in 9 volumes of DMEM-FBS with a zirconia bead (5 mm in diameter) at 3,000 rpm for 0.5 min by use of a bead cell disrupter (Micro Smash MS-100, TOMY Seiko, Tokyo, Japan). Each piece (20-50 mg) of duodenum, jejunum (small

intestine 3-5 cm below stomach), cecum, and rectum was washed by mild vortexing in 0.5 ml of DMEM-FBS and homogenized in 0.5 ml of new DMEM-FBS with about 50 zirconia beads (1 mm in diameter) 5 times at 3,000 rpm for 0.5 min by use of a Micro Smash MS-100. The supernatant obtained by centrifugation at 3,000 $\times g$ for 5 min at 4°C was diluted 1:100 (feces) or 1:5 (intestines) in DMEM-FBS. Each dilution was inoculated onto a monolayer of RAW264.7 cells in 12-well plates at a rate of 0.3 ml per well and incubated for 2 h at 37°C in 5% CO_2. The inoculum was removed, and the cells were washed twice with PBS. Virus RNA from monolayer cells was extracted by use of a QuickGene RNA cultured cell kit S (KURABO Industries, Osaka, Japan) and an automated extraction system (QuickGene-810, Kurabo). cDNA was synthesized by using ReverTra Ace –α– (TOYOBO, Osaka, Japan) and Random Primer (Toyobo) according to the manufacturer's instructions.

The primer sequences used in PCR are shown in Table 3-1. The target regions to detect MNV-S7 were focused on the ORF1/ORF2 junction region and ORF2 region. The four primers for the two target regions were prepared, and then the primer set for the nested PCR assay to detect the ORF1/ORF2 junction region was selected. To help design these primers, the FastPCR freeware (http://primerdigital.com) was used, and the GenBank accession numbers of the MNV sequences were as follows: DQ223041, DQ223043, DQ223042, DQ911368, EU004663, EU004664, EU004665, EU004666, EU004667, EU004668,

EU004669, EU004670, EU004671, EU004672, EU004673, EU004674, EU004675, EU004676, EU04677, EU004678, EU004679, EU004680, EU004681, EU004682, EU004683, and AY228235. PCR reactions were performed by use of a QIAGEN Multiplex PCR Kit (QIAGEN, Hilden, Germany). In the first PCR, the cDNA transcripts (5 μl) were mixed with 1 μl each of 10 μM MNoVorf1&2-F5 and MNoVorf1&2-R2 primer, 10 μl of 2×QIAGEN Multiplex PCR Master Mix, and 3 μl of Nuclease-free water. The first PCR products (5 μl) and the primer set comprised of MNoVorf1&2-F6 and MNoVorf1&2-R3 were used in the second PCR. Amplification was performed in a total volume of 20 μl using a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) with the following conditions: 95°C for 15 min, followed by 30 cycles of 94°C for 30 s, 60°C for 90 s, and 72°C for 90 s, and a final extension step of 72°C for 10 min. The second PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized under UV light.

To confirm that only viable MNV RNA was recovered by the method described above, 5 μl of MNV stock solution was inactivated by reaction with 45 μl of 1:200 diluted Purelox for 3 min and diluted at 1:1,000 in DMEM-FBS. As a positive control, MNV was reacted with PBS for 3 min and diluted. The dilutions were inoculated onto a monolayer of RAW264.7 cells, and RNA was extracted in the same manner as above. Then, RT-nested PCR was

performed by use of the RNA.

Virus titration of mouse samples

Virus titration was performed by use of the supernatants of intestine and feces homogenates prepared for RNA extraction as described above. DMEM-FBS (0.3 ml) including the supernatant corresponding to 5 mg, 0.5 mg or 0.05 mg of intestine was added to each well. Then, virus titer was determined according to the method mentioned in the ‘‘Plaque assay’’ section. For virus titration of the supernatant of feces homogenate, 0.8 ml of serially diluted (10^{-2} to 10^{-4}) supernatant was added to each well of a 6-well plate seeded with RAW264.7 cells at a density of 2.0×10^{6} viable cells per well. Subsequent procedures were as mentioned in the ‘‘Plaque assay’’ section, except for use of 2 ml of 1.5% Agar Noble for the 1st and 2nd overlays.

RESULTS

To investigate the effect of WAHS on MNV inactivation, I determined the virus titer of MNV after reaction with WAHS by plaque assay and compared the results with the other disinfectants commonly used. In addition, it is well known that HOCl oxidizes organic materials and rapidly loses its disinfection efficacy when it comes into contact with something that oxidizes easily, such as organic materials (Fukuzaki, 2006). To investigate the influence of organic materials on inactivation of MNV by disinfectants, I also performed inactivation of MNV in the presence of emulsion of mouse feces. Table 3-2 shows the results in the absence and presence of feces emulsion. The virus titers after reaction with PBS (control samples) were 4.4×10^6 PFU/ml and 3.9×10^6 PFU/ml in the absence and the presence of feces emulsion, respectively. For 0.5-min reactions in the absence of feces emulsion, WAHS reduced the virus titer by more than 5 $\log_{10}$. Similar to the results of WAHS, 1:200 to 1:800 diluted Purelox showed more than 5 $\log_{10}$ reductions in the virus titer, and even 1:1,000 diluted Purelox reduced the virus titer by 3.4 $\log_{10}$ in the absence of feces emulsion in a 0.5-min reaction. In the absence of mouse feces emulsion, 70% ethanol reduced the virus titer by more than 5 $\log_{10}$ in a 0.5-min reaction. In the case of the presence of feces emulsion, the reduction titers decreased to 2.3 and 2.5 $\log_{10}$ for WAHS in 0.5- and 1-min reactions, respectively. However, WAHS showed more than a 5 $\log_{10}$ reduction in the

virus titer in a 5-min reaction even in the presence of feces emulsion. These results suggested that the virucidal effect of WAHS against MNV was retained for several or more minutes even in the presence of organic materials. The effect of Purelox also dropped to a 2.6 and 1.0 $\log_{10}$ reduction at the 1:600 and 1:1,000 dilutions, respectively, in the presence of feces emulsion in a 0.5-min reaction. In addition, the effect of 1:1,000 diluted Purelox on MNV inactivation was much lower than that of WAHS containing a residual chlorine concentration of 60 ppm, which was equivalent to that of 1:1,000 diluted Purelox. In the presence of mouse feces emulsion, 70% ethanol reduced the virus titer by more than 5 $\log_{10}$ in 0.5-min reactions.

Because WAHS had the important advantage of being drinkable without the need for dilution and showed a virucidal effect on MNV inactivation even in the presence of organic materials, I tried to eliminate MNV from mice already infected with MNV by substituting WAHS for their drinking water. Mice were orally infected with MNV, and their drinking water was changed to WAHS at 1 week post infection. Table 3-3 shows the results of detection of MNV from feces by RT-nested PCR and the virus titer in feces determined by plaque assay. Throughout this study, RT-nested PCR was performed by use of RNA extracted from RAW264.7 cells that were inoculated with the supernatant of homogenate of samples. In addition, no MNV-specific product was detected in RT-nested PCR using RNA

extracted from RAW264.7 cells that were inoculated with inactivated MNV (Fig. 3-1). Therefore, a positive result for an MNV-specific product in RT-nested PCR indicates the presence of infectious MNV in samples. In the RT-nested PCR results, MNV was detected at 1 week post infection, and then MNV was successively positive in feces of all the mice after the drinking water was changed to WAHS at 1 week post infection. The virus titers were also as high as those of the control, whose drinking water was not changed to WAHS, at 5 weeks post infection. On the other hand, the results for the sites of the intestine showed some efficacy of WAHS (Table 3-4). The results of RT-nested PCR for the intestines showed a little difference between WAHS-drinking mice and control mice; that is, MNV was not detected in the duodena of WAHS-drinking mice, while it was detected from 2/3 of control mice. In addition, MNV was detected in the jejunum of only 1/3 WAHS-drinking mice, while it was detected from all control mice. However, MNV was detected in the ceca of all the mice. The virus titers were undetectable in the duodena and jejuna, and the same level was found in the ceca of all the mice examined.

Next, I evaluated the effect of WAHS and 1:6,000 diluted Purelox on prevention of MNV infection in the mouse. Mice drank the disinfectants for 2 or 4weeks and then were placed in the same cage as a mouse infected with MNV. Supply of mice with disinfectants in place of drinking water was continued for 3 weeks after cohabitation. However, MNV was

detected from feces of all the mice by RT-nested PCR at 1, 2, and 3 weeks after cohabitation (Table 3-5).

DISCUSSION

WAHS is composed of a blend of sodium hypochlorite with hydrochloric acid in tap water, with the pH value adjusted to 6.0–6.4 and the residual chlorine concentration adjusted to about 60 ppm. WAHS is reported to have microbiological effects on various microorganisms (Ono *et al*., 2012). The main effective form of chlorine in WAHS is hypochlorous acid (HOCl), as is the case for NaOCl, which is commonly used for disinfection of microorganisms in animal facilities. Unfortunately, NaOCl has some disadvantages such as corrosion of metal, irritant properties, and strong odor (Fukuzaki, 2006). Considering that WAHS has little corrosiveness, irritant properties, and odor, and does not need to be diluted before use, it might be more suitable than diluted Purelox at high concentrations for use in animal racks and animal rooms. Animal facilities also use 70% ethanol as a disinfectant. Ethanol is a good disinfectant but has the disadvantages of flammability and high cost.

I evaluated the virucidal effect of WAHS on MNV inactivation *in vitro* and compared the results with NaOCl and 70% ethanol. Because NaOCl (Purelox) is usually used after dilution, the virucidal effect of several dilutions of Purelox against MNV was evaluated. I also determined the influence of organic materials such as feces on the effect of disinfectants. In the absence of feces emulsion, WAHS showed as high efficacy as 70% ethanol and diluted Purelox at high concentration. However, the effect of WAHS dropped to 2.3 and 2.5 $\log_{10}$ in

the 0.5- and 1-min reactions, respectively, in the presence of feces emulsion. The cause of efficacy reduction is considered to be that HOCl oxidizes organic materials in feces emulsion and rapidly loses its disinfection efficacy. Though 1:1,000 diluted Purelox could not completely inactivate MNV, other dilutions of Purelox with chlorine concentrations higher than 60 ppm showed more than 5 $\log_{10}$ reductions in the virus titer in the 0.5-min reaction in the absence of feces emulsion. However, in the presence of feces emulsion, the virucidal effect of Purelox diluted more than 1:600 was markedly down. The cause of the efficacy reduction of Purelox in the presence of feces emulsion is considered to be same as that of WAHS, because the main effective form of chlorine is HOCl in both WAHS and Purelox. On the other hand, WAHS showed more than 5 $\log_{10}$ reductions in the virus titer in the 5-min reaction even in the presence of feces emulsion, suggesting that the virucidal effect of WAHS on MNV was retained for several minutes or more even in the presence of organic materials. From these results, both hypochlorite-based disinfectants are thought to have definite efficacy with regard to inactivation of MNV in the presence of organic materials. As expected, both in the presence and absence of mouse feces emulsion, 70% ethanol showed more than 5 $\log_{10}$ reductions in the virus titer in the 0.5-min reaction as previously described (Belliot *et al*., 2008). These results indicated that the usual use of 70% ethanol is effective enough to inactivate MNV in animal facilities.

It was reported that chlorination (10-13 ppm) of drinking water greatly reduced the colonization of *Pseudomonas aeruginosa* in the intestine of mice (National Research Council, 1991a). Another researcher showed that chlorinated drinking water, containing 6-8 ppm of available chlorine, cleared mice of infection with *P. aeruginosa* (Homberger *et al*., 1993). Another important advantage of WAHS is that it can be drunk without dilution. Therefore, I tried to eliminate MNV from mice infected with MNV by substituting WAHS for their drinking water. MNV was not detected in the duodenum of WAHS-drinking mice 4 weeks after the mice started to drink WAHS. The detection rate of MNV in the jejunum of WAHS-drinking mice also became lower than that of control mice. However, MNV was detected in the cecum and successively excreted in feces of all the mice examined. These results suggested that the effect of WAHS on MNV was limited to the small intestine. MNV was reported to be excreted in the feces of mice on day 1 post oral inoculation (Goto *et al*., 2009), suggesting the rapid propagation of MNV in the mouse cecum. Therefore, even if WAHS reaches the cecum of the mouse and partly inactivates MNV, the rest of the MNV may rapidly propagate and be excreted in feces.

It is well known that HOCl, the main effective form of chlorine in WAHS, oxidizes organic materials and rapidly loses its disinfection efficacy. So, the HOCl in WAHS is possibly decreased by contact with organic materials in the stomach and intestine, and a

sufficient volume of HOCl to inactivate MNV might be unable to come into contact with MNV in the mouse intestine.

Finally, I investigated whether hypochlorite-based disinfectants could prevent mice from getting infected with MNV. Mice drank WAHS or 1:6,000 diluted Purelox for 2 or 4 weeks and then were placed in a cage with a mouse infected with MNV. RT-nested PCR showed that MNV was excreted in feces of disinfectant-drinking mice as early as 1 week after cohabitation. These results showed that MNV infection was not prevented by drinking of hypochlorite-based disinfectants and suggested that the virucidal effects of these disinfectants were not retained for a long time in the mouse intestine.

Previously, it was reported that sentinel mice excreted MNV in feces after 2 days of cohabitation with a mouse infected with MNV (Goto *et al*., 2009), indicating that MNV rapidly spread to a mouse and propagated in its cecum. In addition, MNV survives in mouse feces stored at room temperature for 2 weeks (Manuel *et al*., 2008) and is supposed to require only a small number of viral particles (less than 100 particles) to initiate infection like human norovirus (Patel *et al*., 2009). The propagation velocity, environmental stability, and infectivity of MNV might cause the difficulty in preventing MNV infection by an intermittent inactivation effect.

Fostering was reported to be effective in preventing neonatal mice from becoming

infected with MNV (Compton, 2008). From the result that MNV was not detected in the ovaries and uteri of MNV-infected mice, embryo transfer and caesarean section are also suggested to be efficient means of eliminating MNV (Goto *et al*., 2009). However, a method of eliminating MNV has not been established, and it is not easy to eradicate MNV from contaminated facilities by the test-and-removal method (Kastenmayer *et al*., 2008). In this study, I tried to eliminate and prevent MNV infection in mice by supplying WAHS or diluted Purelox as drinking water as easy and low-cost methods. Unfortunately, drinking disinfectants was not effective, so it is important to keep the facility environment clean by use of effective disinfectants. Also, animals introduced into facilities should be tested as MNV free by quarantine and periodically confirmed as MNV free by microbiological monitoring using ELISA, IFA (Kitagawa *et al*., 2010), and RT-PCR methods (Hsu *et al*., 2005).

Table 3-1. Primers used to detect MNV by RT-nested PCR

	Primer	Sequence (5' to 3')	Polarity[a)]	Positions[b)]
1st PCR	MNoVorf1&2-F5	CGCTTYGGAACRATGGATGCTG	+	5001–5022
	MNoVorf1&2-R2	AGCCRGTRTACATGGCTGAG	–	5340–5359
2nd PCR	MNoVorf1&2-F6	CGCAGGAACGCTCAGCAGTC	+	5029–5048
	MNoVorf1&2-R3	CRAGRTARGGGTTRAGYCCYG	–	5312–5332

[a)] +, sense; –, anti-sense.
[b)] Nucleotide positions correspond to those of the MNV S7 complete genome (AB435514).

Table 3-2. Virucidal activities of WAHS, Purelox, and ethanol for MNV

Disinfectant (dilution ratio)	pH	Residual chlorine concentration (ppm)	Reaction Time (min)	Reduction in titer (log_{10} PFU/ml)[a)]	
				Feces emulsion	
				Absennce	Presence
WAHS	6.0-6.4	60	0.5	>5	2.3
			1	>5	2.5
			5	>5	>5
Purelox (1:200)	10.2	>200	0.5	>5	>5
			1	>5	>5
			5	>5	ND
Purelox (1:400)	9.7	150-200	0.5	>5	>5
			1	>5	>5
			5	ND[b)]	ND
Purelox (1:600)	9.5	100	0.5	>5	2.6
			1	>5	3.8
			5	ND	>5
Purelox (1:800)	9.4	70	0.5	>5	2.0
			1	>5	2.2
			5	ND	>5
Purelox (1:1,000)	9.3	60	0.5	3.4	1.0
			1	3.8	1.4
			5	4.5	2.2
70% Ethanol			0.5	>5	>5
			1	>5	ND
			5	>5	ND

[a)] MNV titer was determined by plaque assay.
[b)] ND, not determined

Table 3-3. Detection of MNV in feces of mice that drank WAHS[a)] post MNV infection

Drinking water		Weeks post infection				
		1	2	3	4	5
WAHS (n=3)	RT-nested PCR	3/3[d)]	3/3	3/3	3/3	3/3
	MNV titer[c)] (log_{10} PFU/g)	5.29±0.53	3.79±0.34	3.69±1.10	3.89±1.01	3.74±0.82
Control (n=3)[b)]	RT-nested PCR	3/3	3/3	3/3	3/3	3/3
	MNV titer (log_{10} PFU/g)	5.52±0.54	3.51±0.79	3.38±0.57	3.77±1.24	3.95±0.85

[a)] Drinking water was changed from 1 week post infection.
[b)] The drinking water of control mice was not changed to disinfectant.
[c)] MNV titer was determined by plaque assay. Each value represents the mean ± SD of 3 samples. For calculation of the mean ± SD, titers of samples that did not show any plaque were estimated as the detection limit, 3.0 log10.
[d)] Each value represents number of positive samples / number of samples examined.

Table 3-4. Detection of MNV in the intestines of mice that drank WAHS[a)] at 5 weeks post MNV infection

Drinking water		Intestine			
		Duodenum	Jejunum	Cecum	Rectum
WAHS (n=3)	RT-nested PCR	0/3[d)]	1/3	3/3	0/3
	MNV titer[c)] (log_{10} PFU/g)	<2.3	<2.3	2.92±0.56	ND[e)]
Control (n=3)[b)]	RT-nested PCR	2/3	3/3	3/3	0/3
	MNV titer (log_{10} PFU/g)	<2.3	<2.3	2.59±0.76	ND

[a)] Drinking water was changed from 1 week post infection.
[b)] The drinking water of control mice was not changed to disinfectant.
[c)] MNV titer was determined by plaque assay. Each value represents the mean ± SD of 3 samples.
[d)] Each value represents number of positive samples / number of samples examined.
[e)] ND, not determined

Table 3-5. Detection of MNV in feces of disinfectant-drinking mice that cohabited with an MNV-infected mouse by RT-nested PCR

Drinking water	Weeks before cohabitation[a)]	Weeks post cohabitation		
		1	2	3
WAHS (n=3)	2	3/3[c)]	3/3	3/3
Purelox (1:6,000) (n=3)	2	3/3	3/3	3/3
Control (n=3)[b)]	2	3/3	3/3	3/3
WAHS (n=3)	4	3/3	3/3	3/3
Purelox (1:6,000) (n=3)	4	3/3	3/3	3/3
Control (n=3)	4	3/3	3/3	3/3

[a)] The drinking water of mice was changed to WAHS or 1:6,000 diluted Purelox 2 or 4 weeks before cohabitation with an MNV-infected mouse.
[b)] The drinking water of control mice was not changed to disinfectant.
[c)] Each value represents number of positive samples / number of samples examined.

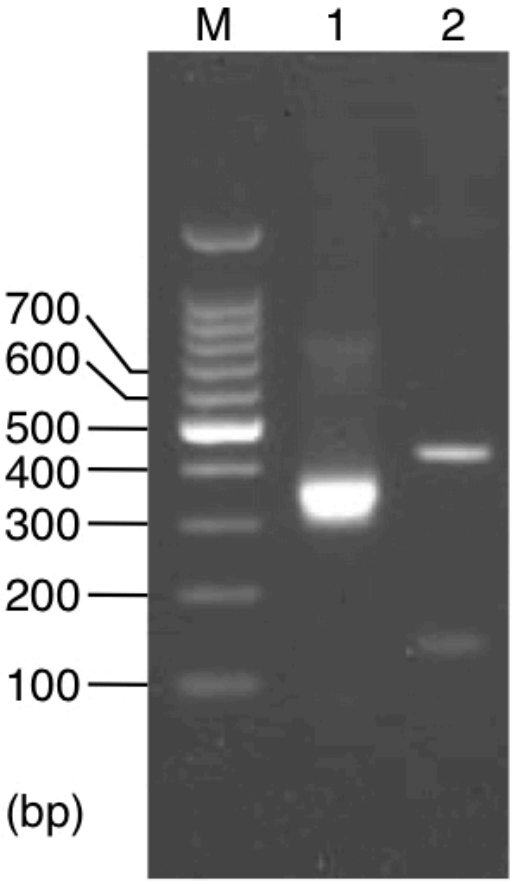

Fig. 3-1. Agarose gel electrophoresis of RT-nested PCR products. Lane M, DNA marker with the sizes indicated; lane 1, MNV-specific RT-nested PCR products (304 bp) as a positive control; lane 2, RT-nested PCR product using RNA extracted from RAW264.7 cells that were inoculated with inactivated MNV.

General Discussion

In the past few decades, circumstances relating to animal experiments have been markedly improved to produce and maintain a healthy environment for laboratory animals and to obtain a more useful and reliable result of research. Currently, use of SPF animals for animal experiment is recognized as usual and the environment of animal facility is protected from pathogenic microorganisms by improved microbiological examination technology, routine cleaning using suitable disinfectants, and equipment like ventilation system supplying clean air through HEPA filter. Meanwhile, there remains the possibility that animal facilities are contaminated with adventitious pathogens that are stored within biological materials in a freezer or liquid nitrogen, that have been comparatively prevalent in foreign countries but hardly found in Japan, or that may be newly isolated from laboratory animals in the future. So, personnel involved in animal experiments should always beware of the microbiological quality of laboratory animals and biological materials derived from animals to prevent animal facility from contamination with pathogenic microorganisms.

In Chapter 1, I indicated the usefulness of ELISA using purified LCMV-NP antigen expressed by recombinant baculovirus by investigating the reactivity of antibody in sera of mice experimentally infected with laboratory strain and recently isolated strain of LCMV. Also, by the reaction of sera with negative control antigen prepared by use of polyhedrin-deleted baculovirus in parallel with positive antigen in ELISA, the check of

LCMV contamination is expected to become more precise. One more advantage is that infectious LCMV is not handled in the course of antigen preparation and serological assay, resulting in prevention of LCMV contamination to human in laboratories and animals in animal facilities.

In Chapter 2, I reported that mouse-adapted influenza virus stock used for an animal experiment in the NIID was contaminated with HVJ. Because the influenza virus was serially passaged in mice by pulmonary infection, the contamination was thought to be caused by HVJ-infected conventional mouse used for influenza virus adaptation in another institute. This instance emphasized that biological materials possess a potential risk to cause a contamination with pathogenic microorganisms in animal facilities. Furthermore, this experience reinforced the necessity and importance to use SPF animals when a biological material including mouse-adapted virus strain is produced and to subject the materials derived from animal to a microbiological examination before subsequent experimental use.

In Chapter 3, I evaluated the virucidal effect of WAHS against MNV *in vitro* and attempted to eliminate MNV from mice experimentally infected with MNV and to prevent mice from becoming infected with MNV by substituting WAHS for their drinking water. Unfortunately, drinking WAHS was not effective in eliminating MNV from mice and protecting mice from MNV infection. However, because the *in vitro* efficacy of WAHS on

inactivation of MNV was indicated even in the presence of organic materials, WAHS is useful to keep animal facility environment clean as same as other common disinfectants.

Route cause of HVJ contamination in the animal facility of the NIID was identified to be the presence of HVJ in the preparation of influenza virus stock. Owing to detection of the origin of the contamination and elimination of HVJ from influenza virus stock, HVJ contamination has not occurred in the NIID facility since then. However, other accidental microbiological contaminations occurred in the NIID facility around the time when HVJ contamination was reported (Takimoto *et al*, 1999). One was contamination of rabbits with *B. bronchiseptica,* which was revealed by regular microbiological monitoring. Nasal swab test suggested that contamination was caused by *B. bronchiseptica*-infected rabbits incorrectly introduced from a commercial breeder. Immediately after the contamination was found, all the rabbits used for the animal experiments were sacrificed. Then, rabbits free of *B. bronchiseptica* have been introduced from another commercial breeder to prevent subsequent *B. bronchiseptica* contamination in the NIID facility. In addition, MHV contamination occurred in mouse breeding colonies introduced from four domestic and foreign institutions. The source of MHV contamination was unfortunately unclear, because mouse colonies were maintained in the animal room for many years and 60 out of 63 mice (95%), each taken randomly from each cage, were MHV-positive when the contamination was revealed. As is

well known, one of major cause of microbiological contamination is introduction of contaminated animals into animal facilities (Nicklas, 1995). So, MHV contamination in the NIID facility was speculated to be caused by animals introduced from other facility. Though the origin of MHV contamination was not clear, subsequent MHV contamination has been fortunately prevented in the NIID facility after all the mice in the room was sacrificed. It was probably because there was no new introduction of the same strains of mice as those of MHV-contaminated mice to the NIID facility thereafter. At present, it is possible that contamination of biological materials such as transplantable tumors and cell lines are screened through commercial service by use of PCR test and cultivation. So, biological materials should be subjected to a microbiological examination before experimental use (Peterson, 2008).

In the NIID facility, regular microbiological monitoring has been monthly performed for 20 years. In order to raise the rate of detection of pathogens, sentinel mice used for microbiological monitoring are exposed to dirty bedding from other mouse cages (Compton *et al*., 2004). By using PCR and IFA as a confirmatory test together with ELISA and cultivation in some cases, I endeavor to detect a microbiological contamination more precisely. When animals are transferred from other facility, submission of health report on microbiological examination performed within 2 months is required before animals are

introduced. In some animal facilities, all mice transferred from other facilities except for commercial breeders are introduced to SPF animal room after mice are cleaned up by embryo transfer or cesarean section to prevent microbiological contamination (Ike *et al*., 2007). However, it is impossible that all animal facilities introduce mice from other facility by use of this method because of the requirements including techniques, personnel, equipment, and costs. Currently, the transfers of genetically modified mice generated in each animal facility of universities and research institutions are increased between animal facilities (Yamamoto *et al*., 2001). Therefore, at least in the case of mice from animal facilities except for commercial breeders, animal facility managers should not blindly accept health report of sentinel mice provided by supplier and might have to perform microbiological examination of a mouse taken from mouse colony introduced to confirm that mice are not contaminated with specific pathogens (Nicklas, 2008). Only after animals without contamination are introduced to an animal facility, control measures such as microbiological monitoring and cleaning with disinfectants will exert the protective effect against adventitious pathogens and prevent animals in animal facility from contaminating with pathogenic microorganisms. In addition, it is important to select suitable control measures for each animal facility depending on its own environment.

From my reports in this study, I emphasize it is important to obtain reliable research results from animal experiments that researchers responsibly use biological materials with correct information of their preparation in animal facilities in which environment is kept clean by the utilization of microbiological examinations and disinfectants.

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