Title	Identification of antigenic peptides derived from B-cell epitopes of nucleocapsid protein of mouse hepatitis virus for serological diagnosis
Author(s)	Asano, Atsushi; Torigoe, Daisuke; Sasaki, Nobuya; Agui, Takashi
Citation	Journal of Virological Methods, 177(1), 107-111 https://doi.org/10.1016/j.jviromet.2011.07.006
Issue Date	2011-10
Doc URL	http://hdl.handle.net/2115/47279
Type	article (author version)
File Information	JVM177-1_107-111.pdf

[Instructions for use](#)

**Identification of antigenic peptides derived from B-cell epitopes of nucleocapsid protein
of mouse hepatitis virus for serological diagnosis**

Atsushi Asano,^{a,b,*} Daisuke Torigoe,^b Nobuya Sasaki^b, and Takashi Agui^b

^aLaboratory of Veterinary Biochemistry, School of Veterinary Medicine, Faculty of
Agriculture, Tottori University, Tottori 680-8553, Japan

^bLaboratory of Laboratory Animal Science and Medicine, Department of Disease Control,
Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

*Correspondence to: Atsushi Asano, Laboratory of Veterinary Biochemistry, School of
Veterinary Medicine, Faculty of Agriculture, Tottori University, Minami-4-101, Koyama-cho,
Tottori 680-8553, Japan; Tel./Fax: +81 857 31 5507; E-mail: atasano@muses.tottori-u.ac.jp

E-mail addresses:

atasano@muses.tottori-u.ac.jp (A. Asano)

dori@vetmed.hokudai.ac.jp (D. Torigoe)

nobsasa@vetmed.hokudai.ac.jp (N. Sasaki)

agui@vetmed.hokudai.ac.jp (T. Agui)

Abstract

Mouse hepatitis virus (MHV) infection is found commonly in laboratory mice and this virus has been known to cause various diseases such as subclinical infection, enteritis, hepatitis, and encephalitis. Serological tests are used commonly to diagnose MHV infection. Complete MHV virions have been used primarily as antigens for serological diagnosis to date. To develop an antigen that is more specific, more sensitive, and easier to prepare for serological diagnosis, the antigenic sites in the MHV nucleocapsid (N) protein were screened in this study. Sixteen antigenic linear sequences in the N protein were found using antisera obtained from mice infected naturally with MHV and a peptide array containing overlapping 10-mer peptides covering the entire N protein. From these antigenic sequences, two synthesized peptides, ILKKTTWADQTERGL and RFDSTLPGFETIMKVL, which were consistent with positions 24–38 and 357–372 of the N protein respectively, were used as antigens in ELISA. Evaluation of ELISA with these peptides revealed that both peptides were specific to anti-MHV antisera. Furthermore, ELISA performed using these peptides was more sensitive than commercial ELISA used for a screening sera from mice infected accidentally to MHV maintained in cages, suggesting that these peptides are useful for serological diagnosis of MHV infection.

Keywords: diagnosis, ELISA, epitope mapping, Mouse hepatitis virus, nucleocapsid protein

1. Introduction

Mouse hepatitis virus (MHV) is an enveloped positive-stranded RNA virus, which belongs to the *Coronaviridae* (de Vries et al., 1997). MHV infection causes various diseases such as subclinical infection, enteritis, hepatitis, and encephalitis (Compton et al., 2004; Haring and Perlman, 2001; Matthews et al., 2002). MHV strains are classified as enterotropic or polytropic. Polytropic strains disseminate from the initial site of replication in the respiratory epithelium to neural, hepatic, vascular, and lymphatic tissues. Replication of enterotropic strains is limited generally to replication within the intestinal tract and these strains cause subclinical infection in adult mice. In contrast, polytropic strains are more likely to cause more severe diseases with clinical symptoms (Baker, 1998). Since MHV is one of the most prevalent viruses in laboratory mice (Jacoby and Lindsey, 1997), it is important to detect this virus in mice colonies by appropriate and sensitive screening tests during routine health surveillance programs.

Serological assays detecting antibodies against pathogens in sera are the most popular screening tests for diagnosis of infection (Compton and Riley, 2001). ELISA and the indirect immunofluorescent assay are the most common serological assays for diagnosis of MHV infection (La Regina et al., 1988; Peters et al., 1979). To date, infected cells or purified viruses are used usually as antigens in serological tests for detecting anti-MHV antibodies. Conversely, cross-reactivity between antibodies present in sera and nonspecific antigens used in the test may yield a false positive result. Therefore, the use of purified antigens such as recombinant proteins and synthesized antigenic peptides derived from pathogens will make serological assays more specific and sensitive. MHV comprises at least three proteins: the

envelope glycoprotein designated as the spike (S) protein, the envelope membrane (M) protein, and the nucleocapsid (N) protein (de Vries et al., 1997). The S protein is involved in cellular receptor binding and membrane fusion (Gallagher and Buchmeier, 2001). The N protein is associated with genomic RNA and plays a role in virus assembly through interactions with the viral RNA and the M protein (Hurst et al., 2005; Verma et al., 2006). The N protein consists of three conserved domains among the strains, which are separated by two variable regions (Parker and Masters, 1990). Several monoclonal antibodies against the S, N, and M proteins were reported previously, indicating that these proteins are immunogenic (Fleming et al., 1983; Kubo et al., 1994; Nelson et al., 2000; Stohlman et al., 1994; Stühler et al., 1991). Therefore, these proteins are potential antigens for serological diagnosis.

In this study, the antigenicity of the peptides derived from the MHV N protein was demonstrated by using anti-MHV antisera and peptide array membrane, on which a set of overlapping peptides covering the entire N protein was synthesized. The results showed that 16 antigenic linear sequences existed in the N protein. In these antigenic sequences, two sites coding amino acids 24–38 (ILKKTWADQTERGL) and 357–372 (RFDSTLPGFETIMKVL) of the N protein are strongly antigenic to anti-MHV antisera derived from mice infected naturally. ELISA performed using these peptides was specific and could detect anti-MHV antisera with higher sensitivity, than a commercial ELISA. These results indicate that these two peptides are useful for serological diagnosis of MHV infection in laboratory mice colonies.

2. Materials and Methods

2.1. Mouse sera.

Sera from C57BL/6 mice infected accidentally with MHV were provided by Prof. Ichiro Miyoshi from the Center for Experimental Animal Science, Graduate School of Medical Sciences, Nagoya City University. As per the guidelines of the Institutional Animal Care and Use Committee (IACUC), Nagoya City University, for screening and eradication of MHV infection, the mice were euthanized and sera were obtained. Normal mice sera were acquired from BALB/c, C57BL/6N, and DBA/2 mice obtained from SLC (Shizuoka, Japan). These sera were derived from the samples obtained for routine screening during a health surveillance program in the animal facility as per the guidelines of IACUC of Graduate School of Veterinary Medicine, Hokkaido University.

2.2. MHV-N peptide array

A set of overlapping 10-mer peptides, obtained by shifting one amino acid from the amino terminus to the carboxy terminus of the N protein that was derived from MHV-JHM strain (GenBank ID: X00990), were synthesized on derivatized cellulose membranes (INTAVIS Bioanalytical Instruments AG, Köln, Germany) using the Auto Spot Peptide Synthesizer ASP-222 (INTAVIS Bioanalytical Instruments AG) according to the manufacturer's protocol (Frank, 2002). Four hundred forty-six peptides were synthesized on the membrane, which are consistent with the peptides coding amino acids from 1–10 to 446–455 of the N protein.

2.3. Detection of antibodies in mouse sera binding to peptides on the peptide array membrane

Sera were diluted to 1:1,000 with 5% skim milk in PBS-Tween 20 (137 mM NaCl, 81 mM Na₂HPO₄, 15 mM KH₂PO₄, 27 mM KCl, and 0.1% Tween 20) and incubated with the peptide array membrane for 2 h at room temperature. The peptide array membrane was washed with PBS-Tween 20 three times and incubated with the HRP-conjugated anti-mouse IgG (GE Healthcare BioSciences, Uppsala, Sweden) diluted to 1:10,000 with 5% skim milk in PBS-Tween 20 for 2 h at room temperature. After reaction with the HRP-conjugated anti-mouse IgG, the membrane was washed with PBS-Tween 20 three times. The antibodies bound specifically to the peptides on the membrane were detected by the ECL Advance Western Blotting Detection Kit (GE Healthcare Biosciences). Visualization and calculation of chemiluminescence of each spot were performed using LAS-3000 imaging system (Fuji film, Tokyo, Japan).

2.4. ELISA

A commercial ELISA, MONILISA MHV (Wakamoto Pharmaceutical Co., Ltd., Tokyo, Japan), was used in this study. Sera used in this ELISA were diluted to 1:40. Evaluation of whether sera were MHV positive or negative was confirmed by comparison with immunoreactivity of positive control sera provided by the manufacture. The peptides coding amino acids 24–38 (ILKKTWADQTERGL) and 357–372 (RFDSTLPGFETIMKVL) of the MHV-N protein were synthesized by Invitrogen (Carlsbad,

CA, USA). In a 96-well plate, 200 µl of 0.5 µM peptide in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6) was seeded, and the plate was incubated overnight at 4 °C. The wells were washed three times with PBS-Tween 20 and incubated with 200 µl of 1% BSA in PBS-Tween 20 (blocking buffer) for 1 h at 37 °C. The wells were washed three times with PBS-Tween 20, and 200 µl of sera diluted to 1:200 with blocking buffer was added and the plate was incubated for 1 h at 37 °C. After washing the well three times with PBS-Tween 20, 200 µl of the HRP-conjugated anti-mouse IgG diluted to 1:10,000 was added to the wells and incubated for 1 h at 37 °C. After washing the well three times with PBS-Tween 20, 200 µl of 1.5 mg/ml *o*-phenylenediamine with 0.01% H₂O₂ was added to each well and incubated for 10 min at 37 °C. Following this, 50 µl of 6 N H₂SO₄ was added and OD was measured at 450 nm using a plate reader.

2.5. Statistics

For statistical analyses, Student's t-test, Fisher's exact test, and chi square test were performed using Statview 5.0 software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Epitope mapping of the MHV N protein

To determine antigenic peptides for detecting anti-MHV antibodies, B-cell epitope mapping of the MHV N protein was performed using the peptide array membrane. This array

membrane comprised 446 spots, which consisted of 10-mer peptides obtained by a single amino acid shift, to produce a complete overlapping set of the N protein. Sera from mice that were infected accidentally with MHV were incubated with the peptide array membrane. These mice were confirmed to be positive using commercial ELISA for detecting anti-MHV antibodies. As shown in Fig. 1, the IgG antibodies in the sera, bound specifically to the peptides on the spots, were detected. The serum used in Fig. 1 contained antibodies that recognized at least four rows of spots, positioned at 27–29, 84–86, 183–187, and 362–366. This result indicates the presence of four antigenic sites in the MHV N protein that are B-cell linear epitopes. In contrast, no significant intensity was observed when sera from control mice were used (data not shown). Sera from seven mice infected with MHV were then analyzed with the same method. The chemiluminescence intensity of each spot was measured, and the mean values are shown in the graph (Fig. 2). Highest intensity was observed around the 28th spot, on which a peptide consistent with amino acids 28–37 of the MHV N protein was synthesized. A similar intensity was observed at the 362nd spot consistent with the intensity observed at the sequence position 362–371. The peptides on the spots with an intensity higher than 2.0 in Fig. 2 were defined as antigenic, and therefore, as B-cell linear epitopes. Sixteen B-cell epitopes in the MHV N protein were found, and their position and sequence are shown in Table 1.

3.2. Development of ELISA to detect anti-MHV antibodies using antigenic peptides derived from the MHV-N protein

The B-cell epitopes of the MHV N protein from MHV seen in Fig. 2 and Table 1

were expected to be antigens for serological diagnosis of MHV infection. To examine whether these epitopes were appropriate for diagnosis, we developed an ELISA using synthesized peptides corresponding to the B-cell epitopes of the MHV N protein. Two 16-mer peptides corresponding to the amino acids 24–39 (MHV-N 24–39) and amino acids 357–372 (MHV-N 357–372) of the N protein were employed as antigens for this ELISA. The sensitivity and specificity of ELISA using the peptide-antigens were examined by normal or MHV-positive mouse sera. When using the MHV-N 24–39 peptide, the OD values of 1:50-diluted MHV-positive sera were higher than those of the diluted MHV-positive sera, and the values decreased as the dilution increased (Fig. 3, upper graph). In contrast, the OD values of normal sera were almost the same as those without sera. In the case of the MHV N 357–372 peptide, a similar result was seen (Fig. 3, lower graph). These results indicate that both the MHV N peptides could be employed as specific antigens for detecting anti-MHV antibodies.

3.3. Evaluation of ELISA using the MHV-N peptides for serological diagnosis of MHV infection

To evaluate whether ELISA using the MHV-N peptides could be employed for diagnosis, its immunoreactivity was examined using sera from the MHV-contaminated colony that comprised mice infected accidentally with MHV and their cage mates. When sera from eight control mice were used, the mean and the SD were as follows: 0.113 and 0.025 for the MHV-N 24–39 peptides, and 0.181 and 0.023 for the MHV–N 357-372 peptides (Table 2). The means and SD of OD values from the 15 mice maintained in the MHV-contaminated

colony are also given in Table 2. The mean OD values of sera from mice in MHV-contaminated colony were significantly higher than that from control mice. Furthermore, these sera were evaluated by both commercial ELISA and ELISA using the MHV-N peptides. As shown in Table 2, the cutoff OD values of ELISA using MHV-N peptides for MHV-positive sera were determined to be the mean + 2 SD from the control mice. All control samples were negative in each ELISA (Table 3). When sera from mice in the MHV-contaminated colony were used, eight of the 15 samples were positive in commercial ELISA. In contrast, 11 samples were positive in the MHV-N 24–39 and 12 samples were positive in the MHV-N 357–372 ELISA. All samples that were positive in commercial ELISA were also positive in ELISA performed using the two peptides (data not shown). Furthermore, the positive/negative frequency was significantly different between the mice in the control and MHV-contaminated colonies when using ELISA using each MHV-N peptides. In contrast, according to the chi-square test results, the positive/negative frequency of sera from mice in the MHV-contaminated colony was not significantly different between commercial ELISA and ELISA using the MHV-N peptides. These results indicate that the ELISA performed using peptides from the N protein is as specific as the commercial ELISA for the detection of MHV infection.

4. Discussion

MHV infection is found commonly in laboratory mice and causes various diseases such as subclinical infection, enteritis, hepatitis, and encephalitis (Compton et al., 2004; Haring and Perlman, 2001; Matthews et al., 2002). Therefore, it is important to monitor

laboratory mice colonies for MHV infection. Serological assays are used generally for the diagnosis of MHV infection (Compton and Riley, 2001; La Regina et al., 1988; Peters et al., 1979). ELISA is used commonly for diagnosis of MHV infection. In the present study, several B-cell epitopes in the MHV N protein were identified using sera from MHV-positive mice and the MHV-N peptide array membrane. In addition, it was demonstrated that ELISA using the antigenic peptides derived from B-cell epitopes of the MHV N protein is useful for detection of anti-MHV antibodies in mouse sera.

The N protein is one of the structural proteins of *Coronaviridae* (deVries et al., 1997). Previous studies indicated that the N protein of *Coronaviridae* is conserved relatively, immunogenic, and expressed abundantly during infection (Daginakatte et al., 1999; Fleming et al., 1983; King and Brian, 1982). Furthermore, a recent study demonstrates that the whole N protein is suitable as an antigen for serological diagnosis using ELISA (Kunita et al., 2011). Therefore, in this study, it was examined whether the peptides derived from the MHV N protein were suitable antigens for diagnosis. SPOT peptide array was used for screening of antigenic epitopes (Frank, 2002; Reineke et al., 2001; Reineke et al., 2002). In this study, the peptide array membrane was prepared that contained a set of nine amino acids overlapping the 10-mer peptides covering the entire N protein. This array was expected to clarify accurately the comprehensive antigenic linear B-cell epitopes in the N protein. In addition, sera from mice infected naturally with MHV were used for identification of antigenic sites in the N protein in live MHV. As expected, several antigenic sites were detected in the N protein (Figs. 1 and 2 and Table 1). Two sites positioned from amino acids 24 to 39 and from amino acids 357 to 373 were more antigenic than the other sites in the N protein. Previous reports demonstrated the location of epitopes of monoclonal antibodies against the N protein

(Stohlman et al., 1994). The epitopes were classified into five regions: amino acids 1–133, 171–196, 231–249, 249–277, and 374–465. These antigenic regions corresponded, in part, to those in the present results in Table 1. Because sera used for epitope mapping in this study were derived from MHV-infected C57BL/6 mice, the same strain used in the study by Stohlman et al., this result is considered to be reasonable. In addition, some epitopes that have not been reported as yet were found in the present result, e.g., amino acids 150–159, 307–325, 335–346, and 357–373. In particular, the 357–373 amino acid segment was a strong antigenic site in this study. The present results indicate that the approach using peptide array was very efficient for screening the antigenic sites in the entire N protein to identify B-cell epitopes. In addition, these sites are not the same as the RNA binding site in the central domain and the interactive sites of the M protein in the highly conserved carboxy-terminal domain among the strains of MHV (Hurst et al., 2005; Nelson et al., 2000; Parker and Masters, 1990).

In this study, two sites corresponding to amino acids 24–39 and 357–372 of the N protein were selected as ELISA antigens. As shown in Fig. 3 and Table 2, ELISA using the MHV-N 24–39 or MHV-N 357–372 peptides showed no nonspecific cross-reaction to normal sera and was able to show specific reaction to anti-MHV antisera. The positive/negative frequency was significantly different between mice from the control and MHV-contaminated colonies when ELISA was performed with each MHV-N peptides (Table 3). Furthermore, from this ELISA, not only all MHV-positive sera but also some MHV-negative sera diagnosed by commercial MHV ELISA were detected to be positive (data not shown). In contrast, the antigen in commercial ELISA used in this study was the complete MHV virus (Kagiyama et al., 1990). These results indicate that the MHV-N 24–39 and MHV-N 357–372 peptides are as specific as the complete MHV virus for serological diagnosis of MHV

infection, and suggest that the ELISA using these peptides is more sensitive than commercial ELISA. In addition, the utilization of peptides as antigens, compared to the complete MHV as used in commercial ELISA, gives us the advantage in quality control, preparation, and economy of ELISA products. However, the positive/negative frequency of sera from mice in the MHV-contaminated colony was not significantly different between commercial ELISA and ELISA using the MHV-N peptides. In addition, the reactivity of sera from various mice strains was not examined by ELISA using the MHV-N peptides. Further studies are necessary for validation of sensitivity and specificity of the two peptides to sera from mice in the MHV-contaminated colony.

The sequence of the three carboxyl-terminal residues in the MHV-N 24–39 peptide (GLN) is polymorphic among the strains of MHV. For example, this sequence is substituted into GPN in polytropic MHV-1, MHV-3, and MHV-A59 strains and into AGN in polytropic MHV-S and enterotropic MHV-Y strains (Parker and Masters, 1990). Therefore, it is necessary to examine whether this peptide is reactive to antisera against various MHV strains. In contrast, the amino acids in the MHV-N 357–372 peptide are conserved completely among all MHV strains that protein sequence data were registered in GenBank database, suggesting that the MHV-N 357–372 peptide can detect infection caused by various strains of MHV.

In summary, 16 linear B-cell epitopes were identified in the MHV N protein in this study. The present results indicate that two peptides, ILKKTTWADQTERGLN and RFDSTLPGFETIMKVL, are useful for serological diagnosis of MHV infection using serological assays such as ELISA. Immunological assay with peptide array membrane and antisera is an effective method for identifying B-cell epitopes in the proteins derived from the pathogenic agents and for the development of specific antigens for serological diagnosis.

References

- Baker, D., 1998. Natural pathogens of laboratory mice, rats, and rabbits and their effects on research. *Clin. Microbiol. Rev.* 11, 231–266.
- Compton, S.R., Ball-Goodrich, L.J., Johnson, L.K., Johnson, E.A., Paturzo, F.X., Macy, J.D., 2004. Pathogenesis of enterotropic mouse hepatitis virus in immunocompetent and immunodeficient mice. *Comp. Med.* 54, 681–689.
- Compton, S.R., Riley, L.K., 2001. Detection of infectious agents in laboratory rodents: traditional and molecular techniques. *Comp. Med.* 51, 113–119.
- Daginakatte, G.C., Chard-Bergstrom, C., Andrews, G.A., Kapil, S., 1999. Production, characterization, and uses of monoclonal antibodies against recombinant nucleoprotein of elk coronavirus. *Clin. Diagn. Lab. Immunol.* 6, 341–344.
- de Vries, A.A., Horzinek, M.C., Rottier, P.J., de Groot, R.J., 1997. The genome organization of the Nidovirales: similarities and differences between Arteri-, Toro-, and Coronaviruses. *Semin. Virol.* 8, 33–47.
- Fleming, J., Stohlman, S., Harmon, R., Lai, M., Frelinger, J., Weiner, L., 1983. Antigenic relationships of murine coronaviruses: analysis using monoclonal antibodies to JHM (MHV-4) virus. *Virology* 131, 296–307.
- Frank, R., 2002. The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports—principles and applications. *J. Immunol. Methods* 267, 13–26.
- Gallagher, T., Buchmeier, M., 2001. Coronavirus spike proteins in viral entry and pathogenesis. *Virology* 279, 371–374.
- Haring, J., Perlman, S., 2001. Mouse hepatitis virus. *Curr. Opin. Microbiol.* 4, 462–466.

1 Hurst, K.R., Kuo, L., Koetzner, C.A., Ye, R., Hsue, B., Masters, P.S., 2005. A major
2 determinant for membrane protein interaction localizes to the carboxy-terminal
3 domain of the mouse coronavirus nucleocapsid protein. *J. Virol.* 79, 13285–13297.

4 Jacoby, R., Lindsey, J., 1997. Health care for research animals is essential and affordable.
5 *FASEB J* 11, 609–614.

6 Kagiya, N., Takakura, A., Terada, E., Sakurai, Y., Suzuki, H., 1990. Studies on the
7 development of an ELISA kit for microbiological monitoring. 1. Evaluation of the
8 reliability of the prototype kit by field tests. *Exp. Anim.* 39, 89–96.

9 King, B., Brian, D.A., 1982. Bovine coronavirus structural proteins. *J. Virol.* 42, 700–707.

10 Kubo, H., Yamada, Y., Taguchi, F., 1994. Localization of neutralizing epitopes and the
11 receptor-binding site within the amino-terminal 330 amino acids of the murine
12 coronavirus spike protein. *J. Virol.* 68, 5403–5410.

13 Kunita, S., Kato, K., Ishida, M., Hagiwara, K., Kameda, S., Ishida, T., Takakura, A., Goto, K.,
14 Sugiyama, F., Yagami, K., 2011. Simultaneous detection of antibodies to mouse
15 hepatitis virus recombinant structural proteins by a microsphere-based multiplex
16 fluorescence immunoassay. *Clin. Vaccine. Immunol.* 18, 758–766.

17 La Regina, M.C., Lonigro, J., Woods, L., Hall, W.C., Doyle, R.E., 1988. Comparison of
18 Track XI fluorometric immunoassay with Bio-EnzaBead enzyme-linked
19 immunosorbent assay for detection of serum antibody to mouse hepatitis virus. *J. Clin.*
20 *Microbiol.* 26, 573–575.

21 Matthews, A.E., Weiss, S.R., Paterson, Y., 2002. Murine hepatitis virus, a model for
22 virus-induced CNS demyelination. *J. Neurovirol.* 8, 76–85.

23 Nelson, G.W., Stohlman, S.A., Tahara, S.M., 2000. High affinity interaction between

nucleocapsid protein and leader/intergenic sequence of mouse hepatitis virus RNA. *J. Gen. Virol.* 81, 181–188.

Parker, M.M., Masters, P.S., 1990. Sequence comparison of the N genes of five strains of the coronavirus mouse hepatitis virus suggests a three domain structure for the nucleocapsid protein. *Virology* 179, 463–468.

Peters, R.L., Collins, M.J., O’Beirne, A.J., Howton, P.A., Hourihan, S.L., Thomas, S.F., 1979. Enzyme-linked immunosorbent assay for detection of antibodies to murine hepatitis virus. *J. Clin. Microbiol.* 10, 595–597.

Reineke, U., Ivascu, C., Schlieff, M., Landgraf, C., Gericke, S., Zahn, G., Herzel, H., Volkmer-Engert, R., Schneider-Mergener, J., 2002. Identification of distinct antibody epitopes and mimotopes from a peptide array of 5520 randomly generated sequences. *J. Immunol. Methods* 267, 37–51.

Reineke, U., Volkmer-Engert, R., Schneider-Mergener, J., 2001. Applications of peptide arrays prepared by the SPOT-technology. *Curr. Opin. Biotechnol.* 12, 59–64.

Stohlman, S.A., Bergmann, C., Cua, D., Wege, H., van der Veen, R., 1994. Location of antibody epitopes within the mouse hepatitis virus nucleocapsid protein. *Virology* 202, 146–153.

Stühler, A., Wege, H., Siddell, S., 1991. Localization of antigenic sites on the surface glycoprotein of mouse hepatitis virus. *J. Gen. Virol.* 72, 1655–1658.

Verma, S., Bednar, V., Blount, A., Hogue, B.G., 2006. Identification of functionally important negatively charged residues in the carboxy end of mouse hepatitis coronavirus A59 nucleocapsid protein. *J. Virol.* 80, 4344–4355.

Table 1. Antigenic sequences in the MHV N protein

Position of spots (Intensity > 2.0 in Fig. 2)	Position of amino acids	Peptide sequences
1, 2, 5	1–14	MSFVPGQENAGSRS
24–30	24–39	ILKKTTWADQTERGLN
49–55	49–64	PKQTATTQPNSGSVVP
72, 73	72–82	ITQFQKGKEFQ
81, 83	81–92	FQFAQGQGVPIA
130	130–139	YLGTGPYAGA
150	150–159	WVASQQAETR
165	165–174	VERDPSSHEA
184–186	184–195	VLPQGFYVEGSG
193	193–202	GSGRSAPASR
240–244	240–253	LVLAKLGKDAGQPK
307–316	307–325	SDPQFPILAELAPTAGAFF
335, 337	335–346	KNSGGADGPTKD
347	347–356	VYELQYSGAV
356–364	356–373	VRFDSTLPGFETIMKVLN
384	384–393	GGADVVSPPK

Table 2. Detection of anti-MHV antibody from mice sera by ELISA using the MHV-N peptides

	MHV-N 24–39 peptide			MHV-N 357–372 peptide		
	OD ₄₅₀			OD ₄₅₀		
	mean	SD	mean + 2 SD	mean	SD	mean + 2 SD
Control (n = 8)	0.113	0.025	0.164	0.181	0.023	0.226
MHV-contaminated colony (n = 15)	0.326*	0.205		0.388*	0.219	

* $p < 0.05$ vs control by Student's t-test.

Table 3. Evaluation of ELISA using the MHV-N peptides for detection of MHV infection

	Positive samples/total samples		
	Commercial ELISA	ELISA using MHV-N 24–39	ELISA using MHV-N 357–372
Control	0/8	0/8	0/8
MHV-contaminated colony	8/15 [*]	11/15 [*]	12/15 [*]

^{*} $p < 0.05$ vs control by Fisher's exact test.

Figure Legends

Fig. 1. Detection of antigenic sites in the MHV N protein using a peptide array membrane. Antibodies in mouse sera bound to each peptide on a peptide array membrane were visualized by chemiluminescence. A typical image from seven MHV-positive sera samples is shown.

Fig. 2. Antigenicity of the MHV N protein. Peptide-binding antibodies in sera from mice infected naturally were detected as shown in Fig. 1. The intensity of chemiluminescence at each spot was quantified. The values are relative to the background and are presented as mean $\pm$ SEM of seven sera. The values on X-axis indicate the position of the spots.

Fig. 3. ELISA using the MHV-N peptides. MHV-positive or -negative sera were diluted and used for ELISA using the MHV-N peptides as described in Materials and Methods. The upper graph is a plot of OD₄₅₀ from ELISA using the MHV-N 24–39 peptide. The lower graph is a plot of OD₄₅₀ from ELISA using the MHV-N 357–372 peptide. Open circles indicate MHV-negative sera, and closed squares indicate MHV-positive sera. (-) indicates the sample without serum. The plots are representatives from three independent experiments.

FIGURE 1

FIGURE 2

FIGURE 3

Supplemental Figure