Simultaneous Detection of Antibodies to Mouse Hepatitis Virus Recombinant Structural Proteins by a Microsphere-Based Multiplex Fluorescent Immunoassay

RUNNING TITLE: rMHV ANTIGEN-BASED MULTIPLEX FLUORESCENT IMMUNOASSAY

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We report a new microsphere-based multiplex fluorescent immunoassay (MFI) using recombinant MHV proteins to detect antibodies to coronaviruses in mouse and rat sera. All the recombinant proteins, including nucleocapsid (N) and 3 subunits of spike protein, S1, S2, and Smed, showed positive reactivity in MFI with mouse antiserum to 4 MHV strains (MHV-S, A59, JHM, and Nu67) and rat antiserum to a strain of sialodacryoadenitis virus (SDAV-681). The MFI was evaluated for its diagnostic power with panels of mouse sera classified as positive or negative for anti-MHV antibodies by ELISA using MHV virion antigen and indirect fluorescent antibody assay. The reactivities of 236 naturally infected mouse sera were examined; 227 samples were positive by MFI using S2 antigen (96% sensitivity) and 208 samples were positive using N antigen (88% sensitivity). Based on the assessment by MFI using S2 and N antigens, only 3
serum samples showed double negative results, indicating a false negative rate of 1.3%. In 126 uninfected mouse sera, including 34 ELISA false positive sera, only 7 samples showed false positive results by MFI using either S2 or N antigen (94% specificity). Similarly, the S2 and N antigen-based MFI was 98% sensitive and 100% specific in detecting anti-coronavirus antibodies in rat sera. Thus, this MFI-based serologic assay using the S2 and N antigens promises to be a reliable diagnostic method representing a highly sensitive and specific alternative to traditional ELISA for detecting coronavirus infections in laboratory mouse and rat colonies.

INTRODUCTION

Mouse hepatitis virus (MHV) is one of the most prevalent infectious agents of laboratory mice. At present, the most widely used methods for diagnosis of MHV infections are serological tests by enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody assay (IFA). Although IFA is highly specific, it is subjective and is not suitable for screening of large numbers of samples. ELISA has better throughput but requires larger amounts of serum samples than IFA. Moreover, MHV virion antigen currently used in ELISA is
propagated in mouse cell lines, but purified viral antigens have some disadvantages, such as poor yields of purified viruses, less reproducibility of antigens, and contamination with cellular proteins, resulting in difficulties in quality control of antigens and frequent false positive results in serological tests.

The recombinant antigen-based antibody detection tests are well known to provide high reproducibility, are easy to standardize, and do not require cultivation of infectious agents. Although the use of recombinant antigens in serological tests has been widely reported for a variety of infectious diseases (1, 4, 12, 13, 16, 17, 19, 21, 23), no serological test using recombinant MHV antigens is currently available. MHV is composed of three major structural proteins: nucleocapsid protein (N), spike protein (S), and membrane protein (M). The N, S, and M proteins all contribute to inducing host immune responses. The N protein is the most abundant protein in MHV virion with an amino acid sequence that is highly conserved among MHV strains. The S protein is the major target of the neutralizing antibodies and induces the earliest antibody responses (2, 5, 10, 18). Therefore, we chose N and S proteins as candidates to generate recombinant antigens for the detection of antibody responses to MHV infections.
The microsphere assay technology developed by Luminex Corp. is suited to a high-throughput immunoassay for simultaneous detection of antibodies to multiple pathogens and antigens (4, 7, 8, 9, 11, 24, 25). In microsphere-based multiplex fluorescent immunoassay (MFI), microspheres function as the solid phase to which protein antigens from various pathogens are bound. One hundred sets of color-coded beads are used and each bead set is distinguishable by its fluorescent emission when excited by a classification laser. As each of the bead sets can be conjugated with a unique antigen, each bead represents a separate antibody reaction after incubation with the serum samples to be tested, followed by a fluorescently labeled detection reagent and fluorescent emission by a detection laser. Another distinct advantage of the MFI method is that a trace amount of serum sample is sufficient for multiplex detection.

Here, we report the usefulness of the MFI method using recombinant N and S antigens in serological diagnosis of MHV infections. The sensitivity and specificity of recombinant antigen-based MFI were evaluated in comparison with those of the cell culture virion-based ELISA and IFA using experimentally and naturally infected mouse sera. Furthermore, our MFI system was applied to
serological testing of coronavirus infections in rats including sialodacryoadenitis virus (SDAV) and rat coronavirus (RCV).

MATERIALS AND METHODS

Virus and cells
MHV-S strain was obtained from ATCC and propagated in mouse astrocytoma cell line (DBT) (6). DBT cells were grown in Eagle’s minimum essential medium (MEM) containing 10% fetal bovine serum at 37°C with 5% CO₂ in a humidified incubator.

Animals and serum samples
Antisera to MHV-S, A59, JHM, and Nu67 (20) strains were prepared from 7-week-old female ICR mice inoculated oronasally with 1 × 10⁶ to 1 × 10⁷ TCID₅₀/100 μL of MHV culture stock. Antiserum to SDAV-681 was prepared from 7-week-old female Wistar rats oronasally inoculated with 1 × 10⁶ TCID₅₀/100 μL of SDAV-681 culture stock. At 4 weeks postinfection, serum was collected from the experimentally infected mice and rats euthanized by isoflurane inhalation. For serial serum collection, serum samples were obtained from the tail vein of mice on days 0, 7, 14, 21 and 28 after inoculation with MHV-S. The animal
studies were approved by the institutional animal care and use committees of
the University of Tsukuba and the Central Institute for Experimental Animals.

Uninfected control sera were collected from ICR, C3H, C57BL/6, and
BALB/cA mice, and Wistar rats purchased from CLEA Japan Inc. (Tokyo, Japan)
at 6 to 30 weeks old. Their source colonies were specified to be free of MHV or
SDAV/RCV and the sera collected were confirmed to be negative for MHV or
SDAV/RCV by ELISA and IFA as described below.

To evaluate the specificity and sensitivity of the MFI method for detecting
antibodies in mice and rats naturally infected with MHV or SDAV/RCV, mouse
and rat sera from a variety of sources submitted to the Central Institute for
Experimental Animals for health screening were tested by the MFI as well as
ELISA and IFA for detecting anti-MHV or anti-SDAV/RCV antibodies.

Viral RNA preparation and RT-PCR amplification

DBT cells were infected with MHV-S and incubated for 2 days. The
infected cells were collected and RNA was extracted with RNAiso Plus (Takara
Bio, Otsu, Japan). For cDNA synthesis, aliquots of 100 ng of RNA were
incubated at 65°C for 5 min with 0.5 µM of reverse oligonucleotide primer. After
chilling on ice, 200 units of SuperScript III reverse transcriptase (Invitrogen,
Carlsbad, CA) was added and incubated at 50°C for 1 h in the presence of 0.5 mM each of dNTPs, 5 mM dithiothreitol, and 1× first strand buffer in a reaction volume of 20 µL. Aliquots of 2 µL of cDNA reaction were added to PCR mixtures containing 0.6 µM each of forward and reverse primers, 0.2 mM of each dNTP, 1× Pyrobest buffer II, and 2.5 units of Pyrobest DNA polymerase (Takara Bio) in a volume of 50 µL. PCR was performed for 35 cycles of 15 s at 94°C for denaturation, 30 s at 60°C for annealing, and 2 min at 72°C for extension using a PCR thermal cycler (TP600; Takara Bio). The sequences of oligonucleotide primers used for RT-PCR were as follows: S1-FW, 5'-CCGGATCCATGTTGTCGTTTTATTT-3'; S1-RV, 5'-TAGTC

GACTCAGTGCAGCGCTGAGCA-3'; S2-FW, 5'-CCGGATCCCTCCTCAG

GTACGGTTTATA-3'; S2-RV, 5'-GGTCGACCTCAATCTCAATTAGTGGATGGA-3'; Smid-FW, 5'-ATGGATCCGGATTTATGATGTCGCT-3'; Smid-RV, 5'-CCGTGCA

CTCATACTTTTTGCTA-3'; N-FW, 5'-CTGAGATTCATGTCTTTTGTTCCTGG

G-3'; N-RV, 5'-GCCGCTCGAGTTACATCATTAGAGTCATC-3'. PCR products were separated by 1% agarose gel electrophoresis. Bands were excised from the gels and the products were purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA).
Cloning and sequencing

Purified PCR products were cloned into the plasmid vector pGEM-T Easy (Promega, Madison, WI) and the sequences were determined using a BigDye PCR sequencing kit (Applied Biosystems, Carlsbad, CA) and ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems). The full region of the N gene was subcloned into the EcoRI and XhoI sites of the expression vector pGEX-5X-1 (GE Healthcare, Little Chalfont, UK). The partial regions of the S gene, S1, S2, and Smid (Fig. 1-A) were subcloned into the BamHI and SalI sites of the expression vector pET28a(+) (Novagen, Darmstadt, Germany). After ligation reaction using Ligation High (Toyobo, Osaka, Japan), *E. coli* BL21 or BL21(DE3) competent cells were transformed with the recombinant plasmids.

**Expression and purification of recombinant proteins**

To produce GST-fused recombinant N protein, transformed *E. coli* BL21 was cultured in 1 L of Luria-Bertani (LB) medium containing 100 µg/mL ampicillin at room temperature. When the culture media had reached OD$_{600}$ of 0.6 – 0.8, isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.8 mM and incubated for an additional 2 h at room temperature. Cultures were placed on ice for 10 min and centrifuged at 2000 × g for 10 min. Next, the
supernatant was removed, and the pellet was suspended in 50 mL of Dulbecco’s phosphate-buffered saline (PBS). Then, Triton X-100 at a final concentration of 1% and dithiothreitol at a final concentration of 1mM were added and the mixture was stored at –80°C until purification. The bacterial suspension was thawed and sonicated on ice 15 times with 10-s pulses. The soluble fraction was collected after centrifugation at 8000 × g for 20 min, then 1 mL of 50% glutathione sepharose 4B equilibrated with PBS was added. After incubation with gentle inversion at room temperature for 1 h, the matrix was transferred to a disposable column and washed with 20 mL of PBS. Protein was eluted 4 times by incubation with 1 mL of 50 mM Tris-HCl (pH 8.2) containing 20 mM glutathione at room temperature for 20 min. Usually, only fractions 1 and 2 were pooled for further use because they showed OD_{280} values of 0.8 or higher. In addition, *E. coli* BL21 carrying the pGEX-5X-1 vector which contains the full length N gene of sendai virus (SeV) was utilized to prepare GST-fused SeV N protein as an irrelevant GST control antigen. To produce His-tagged recombinant S1, S2, and Smid proteins, each transformed *E. coli* BL21(DE3) clone was cultured in 500 mL of LB medium containing 60 µg/mL kanamycin at room temperature. When the culture media had reached OD_{600} of 0.4 – 0.5, IPTG was added to a final
concentration of 0.4 mM and incubated for an additional 4 h at room temperature. Cultures were placed on ice for 10 min and centrifuged at 2000 × g for 10 min. Next, the supernatant was removed, and the pellet was suspended in 25 mL of PBS containing 1% Triton X-100, 1 mg/mL lysozyme, and 1 mM dithiothreitol, and the mixture was stored at −80°C until purification. The bacterial suspension was thawed and sonicated on ice with 30-s pulses 10 times. The insoluble fraction was collected by centrifugation at 8000 × g for 20 min, and the pellet was suspended in 2.5 mL of PBS containing 1 M urea and 1% Nonidet P-40. After sonication with 30-s pulses 10 times on ice, the pellet was collected by centrifugation at 13000 × g for 20 min, resuspended in 2.5 mL of PBS containing 1 M urea, and sonicated with 30-s pulses 10 times on ice. The pellet was then collected again by centrifugation at 13000 × g for 20 min, dissolved in 500 µL of PBS containing 8 M urea, and sonicated with 30-s pulses 10 times on ice. After centrifugation at 13000 × g for 10 min, the supernatant was collected as partially purified recombinant proteins. The concentration of the recombinant proteins was determined by the Bradford protein assay (BioRad Laboratories, Hercules, CA). To assess the quality of the recombinant protein, protein samples were subjected to 8% SDS-PAGE and stained with BioSafe Coomassie (BioRad).
Immunological reactivity of the recombinant protein was confirmed by immunoblotting analysis with mouse antiserum to MHV-S strain (1:1000 dilution), HRPO-conjugated anti-mouse IgG (1:10000 dilution; GE Healthcare), and an ECL Advance Western Blotting Detection Kit (GE Healthcare).

**Coupling of viral proteins to fluorescent microspheres**

Carboxylated fluorescent microspheres (Luminex, Austin, TX) were suspended in 80 µL of 0.1 M NaH₂PO₄ (pH 6.2) and activated by adding 10 µL each of 50 mg/mL N-hydroxysuccinimide (Pierce, Rockford, IL) and 50 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce). The microsphere mixture was incubated for 20 min at room temperature in the dark, centrifuged at 8000 × g for 2 min, and washed twice with 250 µL of PBS. Then, recombinant N antigen and inactivated MHV virion antigen were coupled to microspheres by incubation for 2 h at room temperature in 500 µL of PBS using a rotator in the dark. Recombinant S1, S2, and Smid antigens were coupled to microspheres by incubation for 2 h at room temperature in 500 µL of PBS containing 8 M urea using a rotator in the dark. Antigen-coupled microspheres were washed three times with 500 µL of 0.05% Tween 20 in PBS and resuspended in 250 µL of 1% BSA, 0.05% sodium azide, and 1× Complete Mini protease inhibitor (Roche,
Basel, Switzerland) in PBS. After blocking, the microspheres were stored at 4°C in the dark.

**Microsphere fluorescent immunoassay (MFI)**

MFI was performed to detect antibodies to coronaviruses in mouse and rat sera. Approximately 2000 beads each of antigen-coupled microsphere sets were added to each well of a multiscreen HTS 96-well filter plate (Millipore, Bedford, MA). Beads were washed with 200 µL per well of 0.05% Tween 20-PBS by removing the fluid using a plate vacuum manifold (Millipore). Serum samples were diluted with 1% BSA-PBS and 100 µL was added to each well. The beads were incubated on a plate shaker in the dark for 1 h at room temperature. After incubation, the beads were washed twice by adding 200 µL of 0.05% Tween 20-PBS, shaking for 1 min on a plate shaker, and removing the fluid with a plate vacuum manifold. Next, the beads were resuspend in 100 µL of biotinylated anti-mouse IgG (H+L) (Vector Laboratories, Burlingame, CA) diluted 1:2000 or biotinylated anti-rat IgG (H+L) (Vector Laboratories) diluted 1:1000 in 1% BSA-PBS and incubated on a plate shaker in the dark for 1 h at room temperature. The beads were washed twice with 0.05% Tween 20-PBS as described above, then resuspend in 100 µL of 1 µg/mL
R-phycoerythrin-conjugated streptavidin (Streptavidin-R-PE; Molecular Probes, Eugene, OR) in 1% BSA-PBS. After incubation on a plate shaker in the dark for 30 min at room temperature, the beads were washed three times with 0.05% Tween 20-PBS, resuspend in 100 µL of 0.05% Tween 20-PBS, and analyzed in a Luminex-200 instrument.

**MFI data analysis**

A minimum of 100 gated events were acquired for each bead set by Luminex-200. Results of antibody detection are reported as the median fluorescent intensity (MFI) of R-PE measured from 100 or more counted beads. For each antigen, optimal protein concentration for coupling and cut-off value for designating positive or negative were determined. To set the cut-off value, an average MFI of more than 30 normal mouse or rat sera was calculated for each antigen, and three times the standard deviation value was added to the average. To determine the optimal protein concentration, the signal/noise (S/N) ratio was calculated as the MFI of anti-MHV-S- or anti-SDAV-681-positive control serum diluted 1:100 divided by the cut-off value. Protein concentration at which the highest S/N ratio was obtained was used as the optimal concentration for coupling.
A commercial ELISA kit (Monilisa MHV; Wakamoto Pharmaceutical, Tokyo, Japan) was used to detect anti-MHV mouse antibody and anti-SDAV/RCV rat antibody from 1:40 diluted test sera. Serum samples with OD$_{492}$ values $\geq$ 0.4 were classified as positive by measurement with a microplate reader (model 680; BioRad).

**Indirect fluorescent antibody (IFA) assay**

MHV-Nu67-infected DBT cells were mixed with equal numbers of uninfected DBT cells, spotted onto 12-well Teflon printed slides, fixed in acetone at 4°C for 15 min, and stored at –20°C until use. Aliquots of 10 µL of test sera at a dilution of 1:10 in PBS were added to each well and incubated at 37°C for 15 min. The slides were then washed in PBS and incubated with 10 µL of fluorescein-conjugated anti-mouse IgG (MP Biomedicals, Irvine, CA) or fluorescein-conjugated anti-rat IgG (MP Biomedicals) diluted 1:100 in PBS containing 1% Evan’s blue dye at 37°C for 15 min. After washing the slides, specific fluorescence was analyzed using a UV epifluorescence microscope.

**RESULTS**

15
Cloning of MHV-S strain S and N genes

The N gene sequence obtained was identical to that of the MHV-S strain registered in GenBank with the accession number M35255. The S gene sequence obtained here did not show perfect identity to that of MHV-S strain registered in GenBank with the accession number GU593319, with 4 nucleotide substitutions all resulting in amino acid alterations. The S gene of MHV-S strain showed a high degree of similarity with the S genes of MHV-Y, TY, DVIM, 2 and 1 strains with amino acid identities of 95% to 91%. The degree of S gene similarity was also high between MHV-S and rat coronavirus SDAV-681 and RCV-Parker, with amino acid identities of 92% and 91%, respectively. On the other hand, the degrees of S gene similarity between MHV-S and MHV-3, RI, JHM, or A59 strains were relatively low (amino acid identities of 80% – 82%). The S2 region, *i.e.*, the carboxy-terminal half of the S protein, is highly conserved among MHV strains and rat coronaviruses with identities of 88% to 99%, which were comparable to the degrees of N protein identity (91% – 96%). However, the degrees of identity of S1 region, *i.e.*, the amino-terminal half of S protein, among MHV strains and rat coronaviruses was 73% to 94%, which was lower than that of S2. The complete nucleotide sequence of S gene cloned from the MHV-S
strain has been submitted to DDBJ/EMBL/GenBank under accession No. AB593383.

After sequencing, the full-length N gene was subcloned into the pGEX-5X-1 vector and partial regions of the S gene, S1, S2, and Smid (Fig. 1-A), were subcloned into the pET28a(+) vector. In *E. coli* clones transformed with these recombinant plasmids, 4 recombinant proteins, GST-fused N protein and His-tagged S1, S2, and Smid proteins, were expressed successfully. At first, we attempted to prepare recombinant S protein as a soluble GST-fusion protein in the same way as the N protein. As S protein is too large to be produced efficiently in *E. coli*, we cloned each DNA fragment encoding the N-terminal S1 subunit, which forms the globular head of the peplomer, and the C-terminal S2 subunit, which forms the stalk region. In addition, the middle part of S protein near the proteolytic site, which we designated as Smid, was prepared. As all the truncated recombinant S proteins in the GST-fusion form were expressed in only small amounts, we finally produced the recombinant S1, S2, and Smid proteins as insoluble His-tagged proteins. Unexpectedly, immobilized metal affinity chromatography did not enable to purify a large amount of these His-tagged proteins efficiently. Therefore, we partially purified the insoluble His-tagged proteins.
proteins by washing them in 1M urea solution. Finally, the recombinant S
proteins were solubilized in 8 M urea-PBS and used as antigens for further
analysis.

**SDS-PAGE and immunoblotting analysis of recombinant proteins**

Recombinant proteins produced by *E. coli* were analyzed by SDS-PAGE.

As shown in Fig. 1-B, GST-fused N protein and His-tagged S1, S2, and Smid
proteins were detected as major recombinant protein bands with estimated
molecular weights of approximately 76 kDa, 87 kDa, 68 kDa, and 70 kDa,
respectively. On immunoblotting analysis, all the major recombinant MHV
proteins showed strong reactivity with anti-MHV-S mouse serum (Fig. 1-C).

Several bands that do not correspond to the expected molecular weight of the
recombinant MHV proteins were detected and some of those were
immunoreactive (Fig. 1-B and C). It is likely that the smaller proteins present in
the affinity purified GST-N protein preparation are degraded recombinant N
proteins as those are immunoreactive with anti-MHV serum. Multiple protein
bands present in the His-tagged S protein preparations may be degraded
proteins originated from the recombinant S proteins or *E.coli* proteins
contaminating the insoluble inclusion bodies.
Development of recombinant MHV antigen-based MFI

The reactivities of microspheres coupled with 5 – 40 µg/mL of each recombinant antigen were analyzed using serially diluted anti-MHV-S mouse serum. As shown in Fig. 2, antigen dose-dependent reactions were observed for all of the antigens tested. Although the MFI value to MHV virion antigen decreased sharply at low antigen concentration, all of the recombinant antigens exhibited consistently high MFI values even at the lowest antigen concentration used. Specificity of GST-fused MHV N protein to detect anti-MHV antibody was confirmed by no cross-reactivity of anti-MHV S serum with GST-fused SeV N protein used as an irrelevant GST control antigen (Fig. 2 and Table 2).

Furthermore, linear MFI results across a broad range of antibody concentration were obtained for all of the antigens. This linear reaction property of MFI and low background binding of microspheres allowed MFI to be performed at a serum dilution of 1:100.

The optimal protein concentration of each antigen for mouse serum testing was determined based on reactivity in MFI with 1:100 diluted anti-MHV mouse serum and negative control mouse sera. As shown in Table 1, all the recombinant antigens provided the highest S/N ratio when used at 10 µg/mL;
therefore, the optimal protein concentration was set at 10 µg/mL. The cut-off value to distinguish positive samples was set at 750 MFI for N antigen, 850 MFI for S1 antigen, 800 MFI for S2 antigen, and 1000 MFI for Smid antigen according to the background MFI values calculated as the mean + 3 standard deviation after rounding up to the nearest 50 MFI units. For MHV virion antigen, optimal protein concentration and cut-off value were set at 40 µg/mL and 900 MFI, respectively. In the same manner, antigen concentration and cut-off value were optimized for rat serum testing (Table 1). The optimal protein concentration of S1, S2, and Smid antigens was 10 µg/mL, which was the same in mice and rats, while N antigen was optimal at 5 µg/mL and virion antigen was optimal at 20 µg/mL. The cut-off value was set at 1000 MFI for N antigen, 750 MFI for S1 antigen, 450 MFI for S2 antigen, 500 MFI for Smid antigen, and 600 MFI for virion antigen to measure antibodies in rat sera.

To evaluate cross-reactivity of the recombinant antigens prepared from MHV-S strain, each recombinant antigen and MHV-S virion antigen were analyzed with mouse antisera raised to several MHV strains and rat antiserum to SDAV-681 by MFI (Fig. 3). Antisera to MHV-S, MHV-A59, and MHV-JHM showed a similar reaction pattern. These sera reacted strongly with recombinant N and
virion antigens, moderately with recombinant S2 and Smid antigens, and weakly with recombinant S1 antigen. Antiserum against MHV-Nu67 showed high reactivity with S2 antigen, moderate reactivity with Smid antigen, and weak reactivity with virion, N, and S1 antigens. High cross-reactivity of anti-SDAV rat serum was observed with MHV virion, N, S2, and Smid antigens. These results indicated that the recombinant antigens cross-react with antibodies generated to a variety of MHV strains and SDAV although the strength of reactivity to each antigen varies among viral strains.

A time course of seroconversion to MHV detected by MFI, ELISA and IFA was compared using serially collected serum samples from 10 mice experimentally infected with MHV-S. As shown in Table 2, only 2 or 3 of 10 mice seroconverted to MHV on day 7 after inoculation, and all mice seroconverted on day 14, according to the N-MFI, S2-MFI, virion-MFI and IFA. In contrast, all mice tested positive for anti-MHV antibodies on day 7 in the ELISA. We also found non-specific reactivity with day 0 serum samples in the ELISA analysis, but neither in the MFI nor IFA analysis. These results suggest that ELISA is superior in detecting antibodies to MHV at the early stage of infection and that MFI has high sensitivity and specificity equivalent to IFA.
Sensitivity and specificity of MFI

To evaluate the sensitivity of the recombinant MHV antigen-based MFI,

236 mouse sera naturally infected with MHV and 50 rat sera naturally infected

with SDAV or RCV, confirmed by double positive results in a commercial

MHV-ELISA and IFA, were tested with the MFI. In MHV-infected mouse sera, as

shown in Table 3, the highest sensitivity was obtained with S2 (96%), followed by

Smid (89%), N (88%), virion (76%), and S1 antigen (65%). In SDAV-infected rat

sera, as shown in Table 4, seropositive rates for S2, Smid, and virion were

markedly high with the same sensitivity (98%), followed by N antigen with a

similar high sensitivity (94%), but S1 antigen provided the lowest 60% sensitivity.

With regard to the specificity of the recombinant MHV antigen-based MFI,

MHV-uninfected mouse sera and SDAV/RCV-uninfected rat sera, confirmed by

negative results in IFA, were tested and compared with a commercial

MHV-ELISA (Tables 3 and 4). In MFI for detecting mouse and rat antibodies, all

the antigens revealed high specificity (≥ 90% in mice and 100% in rats). In

contrast, the specificity of a commercial MHV-ELISA was 73% in mice and 90%

in rats.

Figure 4 shows a comparison of S2 antigen-based MFI and N
antigen-based MFI results for 236 sera from mice naturally infected with MHV. Of 236 mouse sera tested, 9 serum samples were negative in S2 antigen-based MFI and 28 serum samples were negative in N antigen-based MFI. Only three serum samples (1.3%) showed double negative results for S2 and N by MFI. Of 126 sera from MHV-uninfected mice, 10 serum samples (7.9%) showed false positive results for either S2 or N antigen. Taken together, these results indicated that the MFI using recombinant S2 and N antigens has ≥ 98% sensitivity and ≥ 92% specificity for detecting MHV and SDAV/RCV antibodies in mouse and rat sera.

DISCUSSION

In this study, the recombinant S and N proteins of MHV produced in *E. coli* were tested as a substitute for purified virion antigens for the detection of antibodies to rodent coronaviruses. A major disadvantage of virion antigen is the variation in purity of virion proteins, which affects the specificity and sensitivity, laborious work to prepare large amounts of antigen, the possibility of remaining infectivity, and loss of reactivity during the inactivation process. First, we prepared the recombinant N protein as a soluble GST-fusion protein. However,
we found that the sensitivity of the N protein-based ELISA was lower than that of the traditional ELISA using virion antigen in a preliminary study. Therefore, we attempted to prepare recombinant S proteins in GST-fused and His-tagged forms. Because S protein is too large to be produced efficiently in E. coli, we prepared truncated molecules of S protein including the N-terminal S1 subunit, the C-terminal S2 subunit, and the middle part, designated as Smid. In previous studies of MHV (3, 15), bovine coronavirus (22), and severe acute respiratory syndrome coronavirus (14, 26), this middle region was identified as an immunodominant linear neutralization domain by analysis using a prokaryotic protein expression system and monoclonal antibodies or patients’ sera. As a result, a large amount of the His-tagged S1, S2 and Smid proteins were produced as insoluble proteins and were solubilized in a high molar urea solution. All of the recombinant S proteins solubilized under denaturing conditions using 8 M urea showed immunoreactivities with anti-MHV antibodies on immunoblotting and MFI. We found that the MFI reactivity of the microspheres coupled with S proteins in 8 M urea-PBS was stronger than that of the microspheres coupled in PBS. These observations indicated that the recombinant S proteins solubilized with 8 M urea were capable of being coupled efficiently to microspheres in 8 M urea.
urea-PBS. These findings may reflect the appearance of insoluble aggregates of the recombinant S proteins in PBS, which leads to a decrease in coupling efficiency and MFI reactivity.

All the recombinant proteins were expressed from the genes of MHV-S strain. Cross-reactivity of these recombinant proteins to detect antibodies to other MHV strains and SDAV rat coronavirus was confirmed by MFI. According to the calibration curves constructed with the experimentally infected sera to MHV strains and SDAV, cross-reactivity levels of the recombinant proteins were compared. The MFI values corresponding to the 4 recombinant proteins were similar among the antisera. However, antiserum to MHV-JHM reacted very weakly with S1 antigen. This weak reactivity was unlikely a consequence of the relatively low amino acid sequence identity (76%) of S1 between MHV-JHM and MHV-S, because the antibody level to S1 in anti-MHV-A59 serum was equivalent to or higher than that in anti-MHV-S serum despite the even lower S1 identity (73%) between MHV-A59 and MHV-S. Furthermore, antiserum to MHV-Nu67 was remarkable for its lower antibody level to N antigen than to S2 and Smid antigens. Such low reactivity to N antigen may be reflective of the slow growth of MHV-Nu67, a low pathogenic strain isolated from nude mice (20). This may be
correlated with the observation that the S protein is the earliest recognized antigen by the host immune system, whereas the N protein elicits antibody responses during the late stage of infection (2, 14). Nevertheless, the possibility that the differences in reaction pattern among the antisera to virus strains described above were simply due to individual variation among the experimentally infected animals cannot be excluded.

The MFI calibration curve of each antigen constructed with anti-MHV-S serum suggested that MFI provides a broad dynamic range of antibody concentration, which allows accurate analysis of antibody levels at a single point of serum dilution. We assessed 32 mouse sera and 30 rat sera from MHV or SDAV/RCV-free commercial breeder animals at 1:100 dilution, and established an optimal antigen concentration and a positive MFI cutoff value corresponding to each antigen. The protein concentration providing the highest S/N ratio was determined as the optimal antigen concentration and the mean MFI + 3SD value was set as a positive MFI cutoff value. According to these criteria, serial serum samples from experimentally infected mice and a large number of serum samples from naturally infected laboratory mice were analyzed to select the favorable antigens in MFI for detecting antibodies to MHV and to evaluate
diagnostic predictability. Experimental infection study showed that seroconversion in the MFI took place from 7–14 days post MHV infection in the majority of infected mice. In contrast, all of the infected mice seroconverted in ELISA by day 7. For early detection of MHV antibodies, ELISA is more sensitive than MFI, whereas the sensitivities of N-based MFI and S2-based MFI are equivalent to that of IFA. Because IFA is the primary method used as confirmatory testing for ELISA and MFI-based screening assays, special attention should be paid to false negative results in MHV antibody detection within 2 weeks after infection. With regard to the reactivity of sera from naturally infected mice, our results indicated that S2 antigen has the highest sensitivity, but still 4% (9 of 236) of samples show false negative results. The recombinant N antigen resembles MHV virion antigen in immunoreactivity with naturally and experimentally infected sera. While the sensitivity of N-MFI was 88%, 6 of 9 false negative samples in S2-MFI showed positive results in N-MFI. These results indicated that multiplex serological screening, consisting of S2-MFI and N-MFI, has 98% sensitivity for detecting MHV infection in mice. Naturally infected animal sera used in this study were retrospective serum samples screened for positivity for antibodies to MHV by ELISA and IFA. Therefore, it is likely that the sensitivity
of MFI is equivalent to that of the traditional ELISA and IFA. Thirty four sera that
were negative for anti-N or anti-S2 antibodies may represent samples collected
from mice at the early stage of infection. It is not likely that the failure of detecting
antibodies based on the MFI using N or S2 antigen resulted from higher serum
dilution in MFI (1:100 dilution) than that in ELISA (1:40 dilution), because no
significant correlation was seen between ELISA OD values and MFI values in
these sera. Many of the singly positive sera showed definite reactivity with either
N or S2. Thus, antibody production to each antigen varies among individuals or
strains of animals as well as virus strains, and consequently multiplex analysis
using polyvalent antigens is recommended in recombinant antigen-based
serologic testing.

Based on the assessment of 126 mouse sera and 59 rat sera from MHV
or SDAV/RCV-uninfected animals, which were confirmed by IFA, we found that
the MFI had a significantly lower false positive rate than ELISA. The lower false
positive rate in MFI may be due to the lower nonspecific binding of serum
antibodies to the microspheres than to ELISA plates (11). In the case of the
recombinant antigen-based MFI, the purified recombinant proteins are
covalently coupled to microspheres and antigen purity eliminates nonspecific
reactions that often cause high background problems in ELISA (25). Furthermore, the linear reaction and high sensitivity allow MFI to be used for analysis of serum samples at a dilution of 1:100. As MHV ELISA was performed using 1:40 diluted sera, the higher serum dilution in MFI definitely affected the low background.

In summary, the MFI method developed with recombinant MHV S2 and N antigens was as sensitive as and more specific than the traditional MHV ELISA for screening antibodies to coronaviruses in mouse and rat sera. MFI format assays have been used for the simultaneous detection of antibodies to a variety of pathogens in laboratory rodents (4, 7, 8). Although MHV virion antigen was successfully used to develop the MFI system for serodetection of 10 mouse pathogens (8), this is the first report of recombinant antigen-based MFI to detect MHV infection. There are many advantages to MFI over ELISA when screening large rodent colonies. MFI allows screening animals for serum antibodies to multiple pathogens or antigens simultaneously in one reaction well for each animal. This markedly reduces the time and labor required to perform a separate ELISA for each antigen. The MFI format also requires much less serum than ELISA, as multiple agents can be screened using only 1 µL of undiluted serum. In this regard, MFI is suitable for serological screening of small laboratory...
animals, such as mice, often through survival bleeding. The amounts of antigens required for coupling microspheres for 200 analytes are equal to those used for coating a single well of an ELISA plate, thereby greatly saving cost, time, and labor for preparing antigens. In our experience, antigen-coupled microspheres can be stored in the refrigerator for at least 6 months without significant reduction in immunoreactivity. The multiplexing feature of the MFI method enables high throughput and automated screening for serological detection of many infectious agents in laboratory animals.

ACKNOWLEDGMENTS

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REFERENCES


Table 1. Determination of the optimal protein concentrations of 5 antigens and cut-off MFI value in mouse and rat serum testing

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Protein concentration of antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40 µg/mL</td>
</tr>
<tr>
<td>N</td>
<td>1550</td>
</tr>
<tr>
<td></td>
<td>(15.4)</td>
</tr>
<tr>
<td>S1</td>
<td>1300</td>
</tr>
<tr>
<td></td>
<td>(5.2)</td>
</tr>
<tr>
<td>S2</td>
<td>2200</td>
</tr>
<tr>
<td></td>
<td>(10.7)</td>
</tr>
<tr>
<td>Smid</td>
<td>3150</td>
</tr>
<tr>
<td></td>
<td>(7.5)</td>
</tr>
<tr>
<td>MHV virion</td>
<td><strong>900</strong></td>
</tr>
<tr>
<td></td>
<td>(26.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Protein concentration of antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1250</td>
</tr>
<tr>
<td></td>
<td>(19.5)</td>
</tr>
</tbody>
</table>

**Background MFI (S/N ratio)**

- **Mouse sera**
  - N: 1550 (15.4)
  - S1: 1300 (5.2)
  - S2: 2200 (10.7)
  - Smid: 3150 (7.5)
  - MHV virion: 900 (26.2)

- **Rat sera**
  - N: 2200 (10.9)
  - S1: 1350 (3.3)
  - S2: 800 (26.2)
  - Smid: 750 (20.3)
  - MHV virion: 1250 (19.5)

**a** By measuring the MFI values of 32 negative mouse sera at a dilution of 1:100, background MFI was defined as the mean + 3 SD after rounding up to the nearest 50 MFI units. Signal/noise (S/N) ratio was calculated as the MFI of 1:100 diluted anti-MHV S strain mouse serum divided by the background MFI. Optimal protein concentration of each antigen was determined at the concentration that provided the highest S/N ratio. The background MFI at the optimal concentration was used as a cut-off MFI value to differentiate between the positive and negative samples (indicated by underlined bold letters).

**b** Optimal protein concentration of each antigen and cut-off MFI value for rat sera were determined in the same manner using 30 negative rat sera.
Table 2. Serial detection of MHV antibodies in sera collected weekly from mice experimentally infected with MHV-S by oronasal inoculation

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFI</td>
<td>0 / 10</td>
<td>2 / 10</td>
<td>10 / 10</td>
<td>10 / 10</td>
<td>10 / 10</td>
</tr>
<tr>
<td>(1:100 diluted sera)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHV-N-MFI</td>
<td>(36±10)</td>
<td>(1050±2090)</td>
<td>(8242±5079)</td>
<td>(9360±5802)</td>
<td>(14753±6748)</td>
</tr>
<tr>
<td>MHV-S1-MFI</td>
<td>0 / 10</td>
<td>0 / 10</td>
<td>4 / 10</td>
<td>5 / 10</td>
<td>8 / 10</td>
</tr>
<tr>
<td>(91±33)</td>
<td>(206±226)</td>
<td>(560±380)</td>
<td>(1176±798)</td>
<td>(1734±815)</td>
<td></td>
</tr>
<tr>
<td>MHV-S2-MFI</td>
<td>0 / 10</td>
<td>2 / 10</td>
<td>10 / 10</td>
<td>10 / 10</td>
<td>10 / 10</td>
</tr>
<tr>
<td>(65±32)</td>
<td>(602±798)</td>
<td>(3148±3009)</td>
<td>(5994±3987)</td>
<td>(13374±5403)</td>
<td></td>
</tr>
<tr>
<td>MHV-Smid-MFI</td>
<td>0 / 10</td>
<td>2 / 10</td>
<td>9 / 10</td>
<td>10 / 10</td>
<td>10 / 10</td>
</tr>
<tr>
<td>(130±54)</td>
<td>(1062±2009)</td>
<td>(2293±1755)</td>
<td>(3328±2019)</td>
<td>(5884±2598)</td>
<td></td>
</tr>
<tr>
<td>MHV-virion-MFI</td>
<td>0 / 10</td>
<td>3 / 10</td>
<td>10 / 10</td>
<td>10 / 10</td>
<td>10 / 10</td>
</tr>
<tr>
<td>(130±101)</td>
<td>(686±724)</td>
<td>(8135±5987)</td>
<td>(11374±7058)</td>
<td>(16553±7114)</td>
<td></td>
</tr>
<tr>
<td>GST control-MFI d</td>
<td>0 / 10</td>
<td>0 / 10</td>
<td>0 / 10</td>
<td>0 / 10</td>
<td>0 / 10</td>
</tr>
<tr>
<td>(338±205)</td>
<td>(363±164)</td>
<td>(383±164)</td>
<td>(356±175)</td>
<td>(433±265)</td>
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</tr>
</tbody>
</table>

ELISA
(1:40 diluted sera)

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHV-ELISA</td>
<td>2 / 10</td>
<td>10 / 10</td>
<td>10 / 10</td>
<td>10 / 10</td>
<td>10 / 10</td>
</tr>
<tr>
<td>(0.125±0.226) c</td>
<td>(0.991±0.538)</td>
<td>(2.863±0.437)</td>
<td>(3.113±0.271)</td>
<td>(3.370±0.093)</td>
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</table>

IFA
(1:10 diluted sera)

<table>
<thead>
<tr>
<th>Days after inoculation</th>
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<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHV-IFA</td>
<td>0 / 10</td>
<td>2 / 10</td>
<td>10 / 10</td>
<td>10 / 10</td>
<td>10 / 10</td>
</tr>
</tbody>
</table>

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^a Number of positive animals / number of animals examined.
^b Mean MFI value ± SD.
^c Mean OD value ± SD.
^d SeV-N-GST protein was used as an irrelevant GST control antigen.
Table 3. Sensitivity and specificity of MFI antigens for detection of MHV antibodies in mouse sera

<table>
<thead>
<tr>
<th>No. of mouse sera tested</th>
<th>MFI</th>
<th>Commercial MHV virion</th>
<th>Commercial MHV ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>S1</td>
<td>S2</td>
</tr>
<tr>
<td>True positive (TP)</td>
<td>236</td>
<td>208</td>
<td>154</td>
</tr>
<tr>
<td>False negative (FN)</td>
<td>28</td>
<td>82</td>
<td>9</td>
</tr>
<tr>
<td>True negative (TN)</td>
<td>119</td>
<td>116</td>
<td>119</td>
</tr>
<tr>
<td>False positive (FP)</td>
<td>7</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Sensitivity (TP/[TP+FN])</td>
<td>88%</td>
<td>65%</td>
<td>96%</td>
</tr>
<tr>
<td>Specificity (TN/[TN+FP])</td>
<td>94%</td>
<td>92%</td>
<td>94%</td>
</tr>
</tbody>
</table>

*a* Naturally infected mouse sera were tested. Positive status of mouse sera was confirmed by IFA testing.

*b* Uninfected mouse sera were tested. Negative status of mouse sera was confirmed by IFA testing.
Table 4. Sensitivity and specificity of MFI antigens for detection of SDAV/RCV antibodies in rat sera

<table>
<thead>
<tr>
<th>No. of rat sera tested</th>
<th>MFI antigens</th>
<th>Commercial virion</th>
<th>MHV ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>S1</td>
<td>S2</td>
</tr>
<tr>
<td>True positive (TP)</td>
<td>50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47</td>
<td>30</td>
</tr>
<tr>
<td>False negative (FN)</td>
<td>3</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>True negative (TN)</td>
<td>59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>False positive (FP)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sensitivity (TP/[TP+FN])</td>
<td>94%</td>
<td>60%</td>
<td>98%</td>
</tr>
<tr>
<td>Specificity (TN/[TN+FP])</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Naturally infected mouse sera were tested. Positive status of mouse sera was confirmed by IFA testing.

<sup>b</sup> Uninfected mouse sera were tested. Negative status of mouse sera was confirmed by IFA testing.
FIGURE LEGENDS

Figure 1. Recombinant MHV proteins expressed in E. coli. (A) Schematic representation of the truncated recombinant MHV S proteins expressed in E. coli. (B) SDS-PAGE analysis of the recombinant proteins on an 8% polyacrylamide gel. Full-length N protein was expressed as GST fusion protein, N-GST (76 kDa). Partial-length S proteins were expressed as His-tagged proteins, S1-His (87 kDa), S2-His (68 kDa), and Smid-His (70 kDa). (C) Immunoblot detection of the recombinant proteins, N-GST, S1-His, S2-His, and Smid-His, with anti-MHV S strain mouse serum. N-GST protein was affinity purified using immobilized glutathione and S1-His, S2-His, and Smid-His proteins were partially purified from insoluble extracts by serial treatment with urea solutions. The arrows indicate the major recombinant protein bands. MWM: molecular weight marker.

Figure 2. Reactivity of the recombinant antigens in microsphere fluorescent immunoassay. Recombinant MHV antigens (N, S1, S2, and Smid), MHV virion antigen and GST control antigen (SeV-N-GST) were coupled onto microspheres at a protein concentration of 5 – 40 µg/mL, and their reactivities with serially diluted anti-MHV S strain mouse serum were analyzed by microsphere fluorescent immunoassay (shown by solid lines).

Dotted lines represent the reactivity of GST control antigen with anti-GST rabbit serum.

Figure 3. Reactivities of recombinant MHV antigens with mouse antisera to MHV strains and rat antiserum to SDAV. Recombinant MHV antigens (N, S1, S2, and Smid) and MHV virion antigen were coupled onto microspheres at the optimal protein concentration for each antigen, and their reactivities with serially diluted mouse antisera to MHV strains and rat antiserum to SDAV were analyzed by microsphere fluorescent immunoassay.
Figure 4. Comparison of MFI results using recombinant S2 and N antigens for mouse sera naturally infected with MHV. Sera from 236 mice naturally infected with MHV were measured by S2 and N antigen-based MFI. The results were plotted as MFI values. Dashed lines indicate cut-off values for positive results.

- ●: S2 and N double positive (202 samples),
- △: S2-positive and N-negative (25 samples),
- □: S2-negative and N-positive (6 samples),
- ○: S2 and N double negative (3 samples)
Figure 1