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A Pitfall in Mouse Norovirus (MNV) Detection in Fecal Samples Using RT-PCR, and Construction of New MNV-Specific Primers

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Abstract: The murine norovirus (MNV), which belongs to the *Caliciviridae* family, is prevalent in laboratory mice. Since this virus affects macrophages and dendritic cells, infected mice are not suitable for immunological investigations, making it important to detect MNV infections accurately. When we tested RNA extracts derived from mouse feces for MNV detection using nested RT-PCR with a set of MNV-specific primers reported by Goto *et al.* (*Exp. Anim.* 58: 135–140, 2009), we found that these primers amplified not only an MNV-specific signal but also amplified a relatively weak signal with a size almost identical to that of the specific signal. Analysis of the nucleotide sequence of this amplified signal revealed that it was at least 98% identical to the *exophosphatase* gene of a commensal bacterium, *Bacteroides vulgatus*. Subsequent analysis showed that the signal amplified with a pair of nested primers was from DNA derived from *B. vulgatus*, which is sometimes present in SPF laboratory mouse feces, and the nested primers used were both partly homologous with the *B. vulgatus* nucleotide sequence. We thus designed a new set of nested RT-PCR primers that was not cross-reactive with the *B. vulgatus* genome. PCR products amplified by the newly designed primers were at least 89.3% identical to the MNV *RNA polymerase* gene in all cases. Our findings demonstrated that the primer set we designed was suitable for detecting an MNV-specific signal without cross-reacting with *B. vulgatus* DNA in mouse feces.

Key words: *Bacteroides vulgatus*, MNV-specific primer, murine norovirus, refinement, RT-PCR

Introduction

The murine norovirus (MNV) is a single-stranded positive-sense RNA virus belonging to the *Caliciviridae* family. This virus is highly prevalent among laboratory mice, and it is symptomless in immunologically normal mice [3, 9, 10, 14, 17, 18, 23]. Karst *et al.* showed that susceptibility to this virus is not substantially increased by deleting the *recombination activating gene 1* (*Rag1*) or *Rag2* gene-dependent specific immunity [7]. Geneti-

cally disrupting the *interferon (IFN) receptor* gene or the *signal transducer and activator of transcription 1* (*Stat1*) gene encoding a crucial signaling factor downstream of the IFN $\alpha\beta\gamma$ receptor induces lethal infection [7, 19], indicating that innate immunity is required for protection against this virus. Lymph node abnormalities have recently been reported in diabetes model mice infected with MNV [16]. Thus, MNV may cause various pathologic changes in disease model mice and immunodeficient mice [12]. Since MNV infects macrophages [7]

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and dendritic cells [21], it is necessary to use MNV-free mice when analyzing the immune system and in experiments using immunodeficient mice.

Serological [5, 6, 9, 18] and RT-PCR assays [2, 5, 6, 10, 14] have been widely used to detect MNV infections. In particular, the use of RT-PCR has shown that mice experimentally inoculated with an MNV strain (MNV-2, MNV-3, or MNV-4) have detectable viruses in the feces from day 2 to week 8 post infection [5], revealing that mice can become persistently infected and shed virus in the feces for prolonged periods. Recently, a real-time RT-PCR assay has been used to detect MNV RNA [1, 4, 11]; this method has many advantages over conventional RT-PCR, including rapid assay time and the possibility of quantitative measurements, but it is not always highly sensitive [11].

In a nested RT-PCR, efficient virus detection requires test material containing a relatively high amount of virus genome that can be readily collected. While the MNV genome can be detected in various tissues, including the small intestine, cecum, large intestine, liver, mesenteric lymph nodes, spleen, and lungs [2, 6, 8, 12, 15], feces are commonly used for MNV testing because they can be collected noninvasively [10, 14, 17]. Nested RT-PCR thus requires a sensitive primer set and no nonspecific reactions. Taking advantage of the relatively small difference in the *RNA polymerase* gene from various MNV strains [6], Goto *et al.* successfully detected MNV by using a nested RT-PCR with primers targeting this region [2].

In the present study, we found that the primer set used by Goto *et al.* [2] to detect MNV yielded both MNV-specific and nonspecific PCR products. The nucleotide sequence of the latter was almost identical to a particular sequence derived from *Bacteroides vulgatus* DNA. Subsequent analysis showed that the nested primers used indeed cross-reacted with *B. vulgatus*-derived DNA, which is commonly found in the SPF laboratory mouse intestinal microbiota [13]. We therefore designed a new set of primers and found that nested RT-PCR using these primers provided high sensitivity in detecting MNV without cross-reacting with *B. vulgatus*. Our findings also indicated that attention should be given to incidental primer cross-reactivity with commensal bacterial DNA in RT-PCR-based MNV detection using fecal samples.

Materials and Methods

Animals

The study was conducted using 145 sentinel mice from our animal facilities. Female ICR (CrIj:CD-1) mice at 6–7 weeks old were purchased from Charles River Laboratories Japan (CRJ) (Yokohama, Japan) for use as sentinels. The microbiological data proposed by CRJ showed the mice to be free of antibodies against MNV, bacterial pathogens, and parasites. These sentinels were housed in open standard cages (S-cages, TPX, 143 × 293 × 148 mm, Charles River Laboratories Japan) using paper bedding (ALPHA-dri, Shepherd Specialty Papers, Watertown, TN, USA). The cages with bedding were autoclaved before use and changed weekly. The mice were kept under a 12:12 h light-dark cycle in an environment with a temperature ranging from 21 to 25°C, a relative humidity of 30–70%, and 10 air shifts/h. The mice were allowed free access to a pelleted rodent diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and reverse osmosis water with 2 ppm sodium hypochlorite added. These sentinels were housed in 30 animal rooms for 6 months.

To monitor the presence of MNV in our mouse colonies, the sentinel mice were exposed once a month to soiled bedding obtained from about 20% of the total mouse cages. During the study period, the sentinel mice in all the rooms were found to be devoid of antibodies to a panel of mouse viruses (mouse hepatitis virus, Sendai virus, ectromelia virus, and lymphocytic choriomeningitis virus) and devoid of *Mycoplasma pulmonis*, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutscheri*, and *Salmonella* spp. All the animal procedures were approved by the Animal Care and Use Committee of the Osaka University Faculty of Medicine.

RT-PCR

Fresh feces were collected from the mice described above. The collected feces were stored at –80°C until use. RNA was extracted from approximately 30 mg of feces using an RNA extraction kit (RNeasy Mini Kit, Qiagen, Tokyo, Japan). Fecal samples were placed in a homogenate tube, and zirconia beads and 600 µl of Buffer RLT included in the RNeasy Mini Kit were added. The tube was then capped, and the sample was homogenized for 5 min (30 cycles) using a TissueLyser from Qiagen (Retsch GmbH, Haan, Germany). Following homogenization, samples were centrifuged for 3 min at

Table 1. Sequences of MNV-specific primers targeting the MNV *RNA polymerase* gene

	Name	Sequence (5'–3')	Location*
Newly designed primers			
1st PCR	rpF1	GAC ATC ATG GTG CGC CTY TCC	4,323–4,343
	rpR1	CTC ATT CAC AAA GAC TGC TG	5,041–5,060
2nd (nested) PCR	rpF2	TGA GGT GGT TTC GAC CAA CC	4,577–4,596
	rpR2	ACC ATT TCT ATC CCA CTA TGG	4,946–4,966
Primes reported by Goto <i>et al.</i>			
1st PCR	1st Forward	GAC ATC ATG GTG CGC CT	4,323–4,339
	1st Reverse	CTC ATT CAC AAA GAC TGC TG	5,041–5,060
2nd (nested) PCR	2nd (nested) F	TCY TTC TAT GGT GAT GAY GA	4,560–4,579
	2nd (nested) R	TCT CAG CAT CCA TTG TTC CA	5,006–5,025

*Based on the nucleotide position of the MNV-1 strain complete genome (accession no. AY228235). The new forward primer (rpF1) is extended by 4 nucleotides to the 5' side compared with that reported by Goto *et al.* [2], whereas the reverse primer (rpR1) is identical to that of Goto *et al.* [2]. The forward primer (rpF2) and reverse primer (rpR2) for the 2nd PCR amplified a 390 bp product.

20,000 × *g*, and supernatants were transferred to another tube. Subsequently, RNA was extracted according to the manufacturer's instructions and stored at –80°C until use. The RNA samples used for the MNV-positive control were extracted from feces of animals tested by the ICLAS Monitoring Center (Kawasaki, Japan) and found to be positive for MNV by the method reported by Goto *et al.* [2], with sequences that were more than 90% similar to the *RNA polymerase* gene of MNV-1. DNA derived from *B. vulgatus* (ATCC 8482 D-5) was obtained from the American Type Culture Collection.

PCR primers directed against the MNV *RNA polymerase* gene used in this study are shown in Table 1. A Reverse Transcriptase M-MLV kit (RNase H free, Takara Bio Inc., Otsu, Shiga, Japan) was used to produce cDNA. Briefly, 12 µl extracted RNA, 1 µl reverse transcriptase, 1 µl Oligo dT primer (Novagen, Madison, WI, USA), 2 µl of 10 mM dNTPs, and 4 µl of the buffer included in the kit were mixed in a tube, and cDNA was generated by heating to 42°C for 30 min and then to 99°C for 5 min. The cDNA was immediately cooled on ice and was stored at –80°C until use. PCR kits (PuReTaq Ready-To-Go PCR Beads, GE Healthcare, Buckinghamshire, UK) were used to detect MNV. The first PCR was performed using 18 µl DEPC-treated distilled water, 2 µl cDNA, and 2.5 µl each of the forward and reverse primers adjusted to a final concentration of 0.25 µM. The PCR mixture was incubated in a thermal cycler (iCycler, Bio-Rad, Hercules, CA, USA). The reaction conditions were the same as those reported by Goto *et al.* [2] (Table 2). The second PCR was performed with

Table 2. Reaction conditions used for nested RT-PCR amplification using a set of new primers

Step	n	Temperature	Duration
1 Denaturation	1	94°C	4 min
2 Cycle	25		
Denaturation		94°C	1 min
Annealing		55°C	1 min
Elongation		72°C	1 min
3 Primer	1		
Elongation		72°C	5 min

These conditions are the same to those reported by Goto *et al.* [2] and were used for first/second PCR using a set of new primers.

2 µl of the first PCR product and 23 µl of the reaction mixture containing the nested primer set (Table 1), again under the reaction conditions described by Goto *et al.* [2]. PCR products were subjected to electrophoresis using a 1.5% agarose gel at 100 V for 30 min.

PCR for detection of *B. vulgatus*

PCR detection of *B. vulgatus* was carried out by the method reported by Wang *et al.* [20]. PCR primers directed against *B. vulgatus* 16S *rRNA* were as follows: 5'-GCATCATGAGTCCGCATGTTT-3', forward, and 5'-TCCATACCCGACTTTATTCCTT-3', reverse. DNA derived from *B. vulgatus* (ATCC 8482 D-5) was used as a control.

Removing DNA from RNA extracts

To avoid nonspecific PCR reactions, DNA was re-

moved from RNA extracts using a TURBO DNA-free Kit (Ambion Inc., Austin, TX, USA). The degradation procedure was carried out according to the manufacturer's instructions, and the test samples were stored at -80°C until use.

Nucleotide sequence analysis

PCR products amplified with MNV or *B. vulgatus* were purified using a QIAquick PCR Purification Kit (Qiagen) and were used as the template for cycle sequencing. Cycle sequencing reactions were performed by using a BigDye Terminator ver. 1.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies Japan, Tokyo, Japan). Sequencing of the PCR products was performed with the same primers as used for PCR amplification. Sequencing reactions were then subjected to nucleotide sequencing using a 3100 Genetic Analyzer sequencer (Applied Biosystems, Life Technologies Japan). The sequences were then aligned and compared with the nucleotide sequence data of various Norovirus and Sapovirus members registered in the GenBank database (<http://www.ncbi.nlm.nih.gov/Genbank/Genbank-Overview.html>), including human Norwalk virus (M87661), Sapporo virus (U65427), and Hawaii calicivirus (U07661); bovine Jena bovine enteric calicivirus (AJ011099); and murine MNV-1 (AY228235), MNV-2 (DQ223041), MNV-3 (DQ223042), MNV-4 (DQ223043), MNV-5 (EF650480), MNV-6 (EF650481), and murine norovirus (AB469416) using the ClustalW software.

Analysis software

BioEdit version 7.0.5.3 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) was used for nucleotide sequence analysis, TreeView version 1.6.6 was used to generate a phylogenetic tree, and ClustalW version 1.83 was used in the phylogenetic analysis.

Antibody testing

Twenty-four sentinel mice housed in 3 MNV-contaminated animal rooms of our animal facilities were euthanized with CO_2 and bled out by cardiac puncture to obtain serum. Feces were also collected from these mice to perform nested RT-PCR for MNV. The results were compared with those of serological tests for MNV, which were carried out by CRJ. In the laboratory, primary tests were conducted using a multiplexed fluorescent immunoassay (MFI), and positives were determined by indirect fluorescence antibody (IFA) assays.

Statistical analysis

The CORREL function in Excel 2003 was used to compute correlation coefficients between data sets from MNV antibodies and PCR test results.

Results

A previously reported MNV-specific primer set detected both MNV-specific and B. vulgatus-derived signals

We performed nested RT-PCR on mouse feces using a primer set targeting the *RNA polymerase* gene that had been previously reported for MNV detection [2]. However, this MNV-specific primer set was found to detect not only MNV-specific signals but also *B. vulgatus*-derived signals as follows. A majority of the samples (ON1–18) already diagnosed as MNV-positive by the ICLAS Monitoring Center using RT-PCR yielded a strong signal with a size of approximately 466 bp (Fig. 1A, lanes 1–18). Sequencing of the 466-bp PCR products excised from the agarose gel confirmed that they were all derived from the *RNA polymerase* gene of MNV. Hereafter, we used these RNA samples as MNV-positive controls for RT-PCR.

Phylogenetic analysis indicated that these sequences fell into the same cluster as those obtained from known MNVs (Fig. 1B). Unexpectedly, however, nucleotide sequences obtained from 2 samples (ON19 and ON20) yielded a faint band of approximately 466 bp (Fig. 1A, lanes 19 and 20); these sequences fell into a completely different cluster, indicating that they were not derived from the norovirus *RNA polymerase* gene (Fig. 1B, ON19 and ON20). Additionally, these samples were obtained from MNV-seronegative animals (data not shown). DDBJ BLAST software analysis indicated that the sequences obtained from the faint band were 98.6% (ON20) and 99.3% (ON19) identical, respectively, to those of the gene encoding the *exophosphatase* gene of *B. vulgatus* (GenBank accession no. CP000139; Fig. 2), suggesting that these MNV-specific primers we had used were cross-reactive with the *B. vulgatus* genome.

We therefor examined whether the primers we used reacted with the *B. vulgatus* genome. Our results suggested that the 3'-end of the MNV-specific primers was partly homologous with the *B. vulgatus* nucleotide sequence. Specifically, a 7-bp-long region (5'-ATGAYGA-3') of the reverse primer and a 14-bp-long region (5'-CATCCATTGTTCCA-3') of the forward primer used for the second PCR were found to be complementary to

Fig. 1. MNV detection using RT-PCR products amplified with a set of primers originally described by Goto *et al.* RNA extracts derived from sentinel mice housed in our animal facilities were used. (A) Gel electrophoresis of PCR products. Lanes: M, 100-bp ladder; 1–22, RNA extracts derived from sentinel mice (ON1–22); 23, water; 24, MNV-positive control. The arrow indicates the PCR product size. (B) Phylogenetic analysis of the nucleotide sequences obtained from the PCR products indicated in the upper panel and from the reference samples (MNV and related viruses). The scale bar indicates an evolutionary distance of 0.1 between sequences.

sequences found in the *B. vulgatus* genome; these sequences are underlined in Fig. 2.

B. vulgatus-derived DNA was frequently found in RNA extracted from feces

We next examined whether *B. vulgatus*-derived DNA existed in feces using a specific set of primers for *B. vulgatus* 16S rRNA. PCR was carried out using 6 RNA extract samples that were derived from MNV-seropositive (Fig. 1, lanes 8 and 11) and MNV-seronegative (Fig. 1, lanes 19–22) mice. In 5 out of 6 RNA extract samples,

we observed a single prominent band with a size of approximately 287 bp (Fig. 3A, upper panel, lanes 2–6). This band was also observed in *B. vulgatus*-derived DNA (lane 7). Only 1 sample out of 6 was negative for this band (lane 1). DNA degradation of RNA extracts by DNase treatment before the RT reaction resulted in a complete loss of this 287-bp band in the feces (Fig. 3A, lower panel). These data supported the hypothesis that *B. vulgatus* DNA that cross-reacted with this MNV-specific primer set was in fact sometimes present in feces, obscuring interpretation of RT-PCR data for MNV detection.

We then tested whether the MNV-specific primer set reported by Goto *et al.* [2] could amplify a signal from samples containing *B. vulgatus*-derived DNA using RT-PCR. As shown in Fig. 3B, these primers yielded both a thin band of approximately 466 bp (upper panel, lanes 3 and 4) and a fuzzy band of a smaller size from purified *B. vulgatus* DNA (upper panel, lane 7). Corresponding to the data shown in Fig. 1, these primers gave a prominent band of 466 bp in 2 fecal samples (ON8 and ON11) obtained from serologically MNV-positive animals (Fig. 3B, upper panel, lanes 1 and 2). They also gave a thin band of a similar size from 2 fecal samples (ON19 and ON20) obtained from serologically MNV-negative animals (upper panel, lanes 3 and 4) and from *B. vulgatus* DNA (upper panel, lanes 7). However, the samples did not amplify any signal from 2 other MNV-seronegative animals (ON21 and ON22; upper panel, lanes 5 and 6). Moreover, when DNA was removed from these RNA extracts using DNase treatment, the weak band obtained from 2 MNV-seronegative mice (ON19 and ON20) and that from *B. vulgatus* DNA were completely abolished (Fig. 3B; lower panel, lanes 3, 4, and 7), whereas the strong band obtained from the 2 MNV-seropositive mice was unaffected (ON8 and ON11, lower panel, lanes 1 and 2). These results demonstrate that the MNV-specific primer set reported by Goto *et al.* [2] cross-reacted with *B. vulgatus* DNA, giving a false-positive 466-bp band in some of the MNV-negative mice. The identity of the small fuzzy band obtained from purified *B. vulgatus* DNA with the primers reported by Goto *et al.* [2] remains unclear.

Development of new primers targeting the MNV RNA polymerase gene

In order to avoid having to treat fecal RNA extracts with DNase, we next sought to develop a new set of

Fig. 2. Nucleotide sequence alignment of a partial *B. vulgatus* sequence encoding the *exophosphatase* gene and sequences derived from the PCR products for MNV (ON19 and ON20). A dot represents the same nucleotide as that found in *B. vulgatus*. Underlining indicates the same nucleotides as those of the second forward or reverse primers described by Goto *et al.*

highly specific MNV primers that would not cross-react with the *B. vulgatus* genome. The new second PCR primers, shown in Table 1, were directed against a less cross-reactive region in the MNV *RNA polymerase* gene and amplified a 390-bp product. The first PCR forward primer was also modified by adding 4 nucleotides (5'-Y TCC-3') to the 3'-end in order to avoid cross-reaction. The PCR conditions were the same as those reported by Goto *et al.* [2].

When these primers were used for nested RT-PCR, a 390-bp product was detected from untreated RNA ex-

tracts obtained from the feces of MNV-seropositive animals (Fig. 4, ON8 and ON11, lanes 1 and 2), while no signal was detected from the RNA samples obtained from the feces of MNV-seronegative, *B. vulgatus*-positive animals (ON19–22, lanes 3–6). Moreover, no 390-bp product was detected from purified, DNase-untreated *B. vulgatus* DNA (lane 7). These results indicated that our new primers specifically amplified only the MNV genome. Supporting this notion, nucleotide sequencing of the 390-bp band of 18 MNV-positive animals revealed 89.3–92.2% identity to the sequence of the MNV-1 *RNA*

Fig. 3. PCR or RT-PCR products obtained from RNA extracts of untreated fecal matter or RNA extracts subjected to DNase treatment for DNA removal and amplified with *B. vulgatus-16S rRNA* primers or MNV-specific primers described by Goto *et al.* Arrows indicate the PCR product size. (A) PCR products amplified with *B. vulgatus-16S rRNA* primers. Upper panel, untreated RNA extracts; lower panel, RNA extracts subjected to DNase treatment for DNA removal before PCR. (B) RT-PCR products amplified with MNV-specific primers. Upper panel, untreated RNA extracts; lower panel, RNA extracts subjected to DNase treatment for DNA removal before reverse transcription. (A, B) Lanes: M, 100-bp ladder; 1 and 2, MNV-seropositive mice (ON8 and ON11); 3–6, MNV-seronegative mice (ON19–22); 7, *B. vulgatus*-derived DNA; 8, water.

Fig. 4. Gel electrophoresis of RT-PCR products amplified with a new set of MNV-specific primers using DNase-untreated RNA extracts. The arrow indicates the PCR product size. Lanes: M, 100-bp ladder; 1 and 2, fecal RNA extracts from MNV-seropositive animals (ON8 and ON11); 3–6, RNA extracts from MNV-seronegative animals (ON19–22); 7, *B. vulgatus*-derived DNase-untreated DNA; 8, water; 9, MNV-positive control.

polymerase gene for the nucleotide sequence and 97.3–99.1% identity for the amino acid sequence (Table 3). In addition, when the RT-PCR data based on the new primers were compared with the data obtained by an MNV antibody test using 24 different sentinel animals housed in 3 MNV-contaminated rooms of our animal facilities, 4 PCR-positive animals were antibody positive, while 19 out of 20 PCR-negative animals were MNV seronegative and 1 was seropositive (Table 4), showing a strong posi-

Table 3. Extent of identity of the 390-bp band to the MNV-1 *RNA polymerase* gene

Sample no.	Sequence identity (%)	
	Amino acid ^{a)}	Nucleotide ^{b)}
ON1	98.2	89.9
ON2	99.1	90.5
ON3	99.1	89.3
ON4	99.1	89.6
ON5	98.2	90.2
ON6	98.2	91.4
ON7	98.2	90.2
ON8	97.3	90.8
ON9	99.1	91.4
ON10	98.2	91.1
ON11	99.1	90.2
ON12	98.2	89.9
ON13	98.2	92.2
ON14	99.1	89.9
ON15	98.2	90.5
ON16	99.1	90.2
ON17	99.1	90.8
ON18	97.3	90.8

^{a)}115 amino acids. ^{b)}349 bp nucleotides. The percentage of the sequence identity between a part of the *RNA polymerase* gene of MNV-1 and samples (Fig. 1, ON1–ON18) was calculated using the BioEdit software as described in Materials and Methods. The sequences were obtained from 390-bp PCR products amplified with our new primers using RNA extract samples.

Table 4. Correlation of the results between RT-PCR and antibody tests for MNV of sentinel mice

RT-PCR ^{b)}	Antibody ^{a)}	
	Positive	Negative
Positive	4	0
Negative	1	19

The results of the antibody tests and RT-PCR were obtained from 24 sentinel mice housed in 3 MNV-contaminated rooms of our animal facilities. ^{a)}Antibody test was performed by CRJ laboratory. ^{b)}Nested RT-PCR was carried out using a newly designed set of PCR primers. Correlation coefficient = 0.872.

tive correlation between the results obtained from the two tests (correlation coefficient=0.872).

Discussion

Here, we report that an RT-PCR-based MNV detection test using a previously reported primer set [2] detected

not only an MNV-specific signal, but also a signal derived from a commensal bacterium, *B. vulgatus*; the nucleotide sequence of the latter signal showed at least 98% identity with that of the *exophosphatase* gene of *B. vulgatus* (Fig. 1, ON19 and ON20). These faint bands disappeared after removing contaminated DNA from fecal RNA extracts (Fig. 3B). While degradation of DNA from RNA extract may help reduce false-positive results, this process may prolong the period of sample preparation. Moreover, because this bacterium is commonly found in the SPF laboratory mouse intestine [13], the primers reported by Goto *et al.* [2] are likely to confound the interpretation of MNV tests using fecal samples, unless the DNA was first removed. Thus, we designed a new primer set that obviates the need for DNA removal before RT-PCR analysis.

Detecting MNV infection has so far depended mainly on antibodies and RT-PCR assays. The latter method has been used frequently because it allows not only early detection of MNV RNA during MNV infection but also identification of the virus origin through nucleotide sequencing of the PCR products [22]. Primers used to detect MNV have been directed against highly conserved nucleotide MNV sequences, such as those representing the *RNA polymerase* gene [2, 6] and ORF1/ORF2 of the MNV genome [10, 14]. On the other hand, the primer set reported by Goto *et al.* [2] was thought to be selectively directed against the MNV *RNA polymerase* gene. Here, we found that this primer set cross-reacted with the *B. vulgatus* genome; the 3'-end of the MNV-specific primers in the second PCR reaction was homologous with the *B. vulgatus* nucleotide sequence, and this homology appeared to make them cross-reactive with *B. vulgatus* DNA, incidentally yielding a signal very similar in size to an MNV-specific PCR product. Although this cross-reactivity may be circumvented by re-evaluating the annealing temperature conditions or altering the amount of primers used, given that *B. vulgatus* is a commensal bacterium [13], the bacterial DNA will inevitably be present in fecal samples at high frequency, and thus, these primers cannot be relied upon unless the DNA is completely removed from the fecal samples. While real-time RT-PCR assays detecting MNV RNA have been reported recently [1, 4, 11], rigorous examination will be required to verify the assay specificity.

We thus designed new MNV-specific primers that have no cross-reactivity with *B. vulgatus*. First, we performed nested PCR with a newly designed second PCR primer

set and the first primer set reported by Goto *et al.* using *B. vulgatus* DNA. Although the nonspecific thin band disappeared, the unidentified small fuzzy band did not disappear (data not shown). Finally, we modified a forward primer for the first PCR (rpF1) reported by Goto *et al.* by adding 4 nucleotides to improve the assay specificity (Table 1). When we used these newly designed primers, we found a 390-bp signal in RNA samples from the feces of MNV-seropositive animals, but not from the feces of MNV-seronegative, *B. vulgatus*-positive animals. We also did not detect a signal from *B. vulgatus* DNA. Nucleotide sequencing of the 390-bp band verified that it was indeed derived from MNV (Table 3). We then compared these results with those obtained by an MNV antibody test using 24 different sentinel animals and found a strong correlation between these 2 tests (Table 4), further strengthening the notion that the newly designed set of primers could be used to detect an MNV-specific signal. It is of note, however, that 1 out of 20 PCR-negative samples was antibody positive; one possible explanation is that the MNV genome passed only temporarily into the feces despite chronic infection of this animal. Hsu *et al.* also reported that MNV genomes could be detected from feces of some but not all MNV-seropositive mice [5].

Collectively, our study indicates a potential pitfall in detecting MNV infection by RT-PCR using fecal samples. The new primer set designed in the present study should be useful for reliable RT-PCR-based MNV detection in fecal samples.

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