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Detection of Murine Norovirus 1 by Using Plaque Assay, Transfection Assay, and Real-Time Reverse Transcription-PCR before and after Heat Exposure▽

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The correlation between the detection of murine norovirus 1 RNA by real-time reverse transcription-PCR and the infectivity by plaque assay before and after heat exposure (80°C) was examined. No correlation was found in the current study. Moreover, heat inactivation had a much stronger detrimental effect on virus infectivity than on the integrity of the viral genome.

Human noroviruses (NoV) and hepatitis A virus (HAV) are the most important causes of food-borne outbreaks in the Western world (13). These viruses exhibit high particle stability and infectivity, and they cause large and frequent outbreaks that impose significant economic burdens (13). Despite numerous efforts, no cell line is yet available for cultivation of human NoV (5). Recently, a 3-dimensional cell culture model was reported that could provide new avenues in the future (22). The detection of human NoV nowadays relies on molecular techniques and on the use of a surrogate that can be grown in a cell line. Until recently, feline calicivirus (FCV) was almost exclusively used as a surrogate. However, FCV is a vesivirus that causes respiratory illness (10). In 2003, a novel NoV infecting mice, murine norovirus 1 (MNV-1), was described (12). Like human NoV, murine NoV are enteric pathogens that are spread by the fecal-oral route. Moreover, these viruses belong to the same genus (24). A recent report comparing the suitability of MNV-1 and FCV as surrogates for studies of NoV stability and inactivation demonstrated that MNV-1 was more acid tolerant than FCV, making it a more suitable surrogate for human NoV (3). Therefore, MNV-1 was chosen as a surrogate for human NoV in the current study. The objective was to examine the correlation between infective virus particles determined by a plaque assay and genomic copies detected by real-time reverse transcription-PCR (RT-PCR) before and after heat treatment. Furthermore, the effect of heat on the integrity of the viral genome was tested through a transfection assay.

MNV-1 is rapidly inactivated by exposure to temperatures higher than 60°C. MNV-1 lysate samples (400 μl), containing around 7 $\log_{10}$ PFU/ml, were subjected to heat treatment in microcentrifuge tubes in a heating block set at 80°C. The MNV-1 lysate was prepared by infecting RAW 264.7 cells with MNV-1.CW1, passage 5, at a multiplicity of infection of 0.05 for 2 days as described elsewhere (23).

The temperature profile was monitored in parallel in 400-μl volumes of cell culture medium. At chosen time intervals, a suspension of the virus lysate was taken from the heating block and immediately stored on ice until all samples were collected and the number of MNV-1 PFU could be analyzed by a plaque assay (23). Untreated virus lysate served as a control. The inactivation profile and the temperature profile are shown in Fig. 1. After 150 s (2.5 min), the internal temperature of the sample had reached 80°C and the MNV-1 titer was reduced by 6.5 $\log_{10}$ units. These data demonstrate that MNV-1 is rapidly inactivated at temperatures of 60°C or higher. The observed inactivation at about 60°C is likely due to structural changes in the capsid. Conformational alterations on the secondary, tertiary, and quaternary protein levels were also observed for Norwalk virus-like particles at temperatures above 60°C (1).

Development of a real-time PCR assay to detect MNV-1. Due to the absence of a robust tissue culture system for human NoV, molecular techniques are used to detect and to investigate the behavior of human NoV. To determine the relationship between the number of genomic copies and infectious units (PFU/ml), a real-time PCR assay for the detection of MNV-1 was first developed. A primer and probe set were selected with Primer Express (Applied Biosystems, Foster City, CA) in the ORF1/ORF2 junction region (Table 1). Eighteen microliters of reaction mixture (GeneAmp buffer II [Applied Biosystems], 2 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates [Invitrogen, Carlsbad, CA], 200 nM each primer, 200 nM probe, 50 nM ROX [Invitrogen], 0.5 U AmpliTaq Gold [Applied Biosystems]) was added to 2 μl of cDNA. The real-time PCR assays were performed in an ABI 7000 system (Applied Biosystems). The amplification profile included 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

A standard curve (Fig. 2) was generated using 10-fold serial dilutions (10^7 genomic copies to 1 genomic copy) of the plas-

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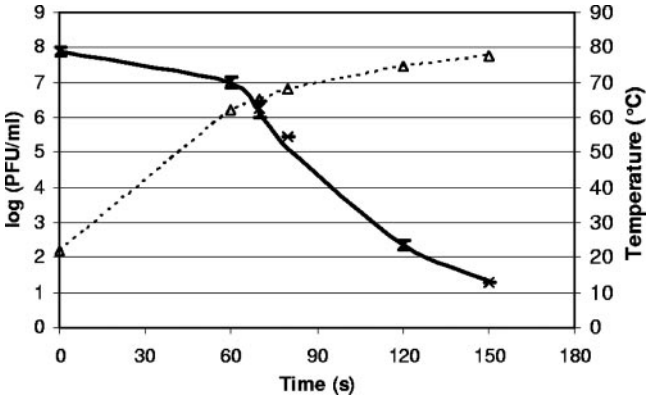

FIG. 1. Inactivation profile of MNV-1 exposed to 80°C (×, solid line), shown as $\log_{10}$ PFU/ml versus time (in seconds). The temperature profile (△, dashed line) is shown as temperature (°C) versus time (in seconds). Experiments were done in duplicate. Error bars, standard deviations (Microsoft Excel, version 2003).

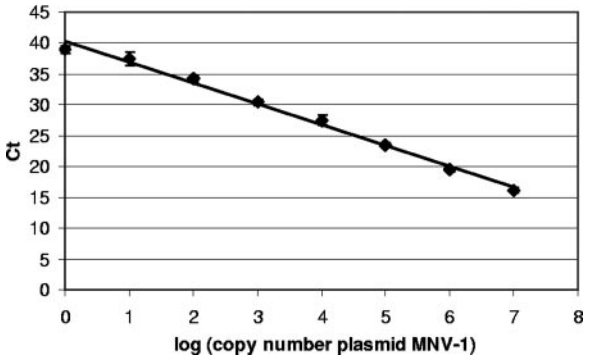

FIG. 2. Standard curve of MNV-1 real-time PCR directed to the ORF1/ORF2 junction region. The cycle threshold (C_T) is plotted against the $\log_{10}$ copy number of the plasmid containing a full-length copy of the MNV-1 genome. Error bars, standard errors of the means (SPSS 12.0 for Windows [SPSS Inc.]) from 10 independent runs.

mid p20.3 containing a full-length cDNA clone of MNV-1.CW1 (21). The standard curve shown in Fig. 2 is a result of 10 independent runs ($y = -3.3865x + 40.31$; $R^2 = 0.99$). A minimum of 10 copies of the MNV-1 genome were consistently detected in the assay, while the detection of 1 genomic copy occurred only one out of three times. This demonstrated that the established assay was sensitive and robust.

Genomic copies of MNV-1 detected by real-time RT-PCR do not correlate with the number of infectious particles after heat treatment. In order to determine whether the number of genomic copies correlates with the number of infectious virus particles after heat treatment, real-time PCR and a viral plaque assay were performed. RNA was isolated from 10-fold serial dilutions of MNV-1 lysate using the RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations, and cDNA was generated. For cDNA synthesis, random hexamers (Invitrogen) were used with Improm-II reverse transcriptase (Promega, Madison, WI) according to the recommended protocol of the supplier in a GeneAmp PCR system 2400 (Perkin-Elmer, Foster City, CA).

The quantification of genomic copies by real-time RT-PCR from 10-fold serially diluted virus lysate samples in the absence of heat treatment demonstrated the presence of ca. 100 times more genomic copies than PFU (data not shown). One reason for this could be the presence of infective virus particles as well as defective or noninfective virus particles in the sample. The presence of distinct virus particles that could serve as a template for real-time RT-PCR detection was also reported by others for Nipah virus and enteroviruses (8, 14).

TABLE 1. Primer and probe set used to detect MNV-1 by real-time RT-PCR

Name	Sequence (5′–3′)[a]	Location
Fw-ORF1/ORF2	CAC GCC ACC GAT CTG TTC TG	4972–4991
Rv-ORF1/ORF2	GCG CTG CGC CAT CAC TC	5064–5080
MGB-ORF1/ORF2	6FAM-CGC TTT GGA ACA ATG-MGBNFQ	5001–5015

[a] 6FAM, the reporter dye 6-carboxyfluorescein; MGBNFQ, minor groove binder with a 3′ nonfluorescent quencher.

The effects of heat exposure on the numbers of infectious MNV-1 particles and genomes were investigated. RNA was isolated directly after exposure of the MNV-1 lysate to 80°C for different times (0, 150, 900, 1,800, and 3,600 s), and cDNA was generated as described above. Exposure of MNV-1 to 80°C for 150 s resulted in PFU reductions of more than 6 $\log_{10}$ units as determined by a plaque assay. At the same time, more than 9 $\log_{10}$ genomic copies were detected by real-time RT-PCR. Even exposure times as long as 1 h at 80°C resulted in the detection of more than 6 $\log_{10}$ genomic copies (Table 2). These data demonstrate that the reduction in the number of infectious virus particles after heat treatment does not correlate with the number of genomes detected by real-time RT-PCR.

Since the use of molecular techniques for the detection of NoV is a common practice in food safety settings, the inclusion of a pretreatment with proteinase K (Prot K) and RNase A prior to RNA extraction was investigated to determine if it could result in a correlation between real-time detection of genomic copies and plaque assay results. A combined treatment of Prot K (20 U) and RNase (100 ng) was reported to degrade viral RNA from inactivated poliovirus particles, while infective virus particles were still detected (18). A Prot K (Sigma, St. Louis, MO) solution of 2 U/μl was prepared in 0.01 M Tris-HCl (pH 7.5). An RNase A (Sigma) stock solution (1 mg/ml) was prepared in 1 M Tris–0.1 M EDTA. The effects of enzymatic activity on an unheated virus lysate (aiming to maintain the integrity of RNA and maintaining RT-PCR detection because infective particles are present as noted by the plaque assay) and on a heat-treated virus lysate (aiming to degrade RNA and eliminate RT-PCR detection in order to obtain a correlation between RT-PCR and the loss of infectivity as noted by the plaque assay) were studied. Both Prot K (20 U/100 μl; 90-min incubation) and RNase A (1 μg/100 μl; 60-min incubation) were tested alone or in combination, at temperatures of 37°C and 56°C, in order to find an effective enzyme treatment (Table 2). Finally the option was taken for the combination of Prot K (30-min) and RNase A (60-min) treatments at 37°C, because this combination reduced significantly (Games-Howell test) and in a representative way (small

TABLE 2. Real-time RT-PCR detection of MNV-1 exposed to heat, showing effects of enzyme treatments prior to RNA extraction

Treatment	Log genomic copies of MNV-1/ml (SD) after exposure to 80°C for:				
	0 s	150 s	900 s	1,800 s	3,600 s
Control (no prior enzyme treatment)[a]	9.39 (0.03)	9.31 (0.02)	9.16 (0.01)	8.77 (0.02)	7.83 (0.08)
Prot K (37°C)	9.36 (0.04)	9.05 (0.02)	8.97 (0.04)	8.69 (0.10)	7.80 (0.03)
RNase A (37°C)	9.30 (0.09)	9.00 (0.19)	8.26 (1.30)	8.12 (0.80)	7.23 (1.05)
Prot K (56°C)	9.37 (0.09)	9.05 (0.12)	8.23 (1.28)	7.81 (1.55)	6.91 (1.63)
RNase A (56°C)	9.20 (0.02)	8.82 (0.09)	7.71 (0.65)	7.06 (0.86)	6.07 (1.43)
Prot K (37°C) and RNase A (37°C)	9.32 (0.14)	9.08 (0.08)	7.58 (0.13)	6.82 (0.09)	6.01 (0.11)
Prot K (37°C) and RNase A (56°C)	9.36 (0.02)	9.11 (0.08)	7.75 (0.46)	6.85 (0.32)	5.91 (0.10)
Prot K (56°C) and RNase A (37°C)	9.35 (0.01)	9.14 (0.07)	7.85 (0.37)	6.56 (0.40)	5.78 (0.39)

[a] The concentrations of infective virus particles, determined by an MNV-1 plaque assay, were 7.87 (standard deviation, 0.13) PFU/ml for exposure to 80°C for 0 s and <1.30 PFU/ml after exposure for 150, 900, 1,800, or 3,600 s.

standard deviations) the number of genomic copies of the heat-treated virus lysate without decreasing the number of genomic copies of the unheated virus lysate. In addition, the combination of enzymes worked synergistically compared to the effects of the enzymes tested separately. As a positive control for RNase A activity, 1 μg enzyme was added to 10 μl of RNA isolated from MNV-1. In this case no genomic copies were detected, proving that RNase A was able to degrade free viral RNA. Moreover, the fact that Prot K reduced infectivity by 3 log units at 37°C (data not shown), while no decline in the number of genomic copies was observed, indicated that Prot K effectively attacked the capsid; no reduction was noticed when the MNV-1 lysate was heated at 37°C without addition of Prot K. The purpose of using Prot K was indeed to attack the protein capsid in order to release the genomic RNA, with the view that unprotected RNA would be more easily broken down, because it would be available for the subsequent RNase A treatment of inactivated virus particles. However, the number of genomic copies of unheated (and thus infective) virus particles should not be affected. Therefore, the concentration of Prot K was not further increased. In summary, these data demonstrate that high numbers of genomic copies (>6 $\log_{10}$) were still detected even after enzyme treatment in the absence of infectious virus. Therefore, no correlation between the number of infectious particles and viral genomes was observed after heat treatment regardless of the presence or nature of the enzyme treatment.

The discussion concerning the correlation between infectivity and the detection of viral genomes has mostly been investigated with regard to viral contamination of water (4, 6, 7). A lack of correlation is known for HAV (11), FCV, poliovirus (7), and adenoviruses (4), demonstrating the limits of the use of molecular techniques in determining the virological safety of water and subsequently also foods. In contrast, others have argued that the detection of genomic equivalents indicates the presence of infectious particles because free RNA is not stable, and therefore the detected genomic copies must originate from encapsidated and thus infectious particles (20). However, the current study represents another demonstration of the limits of detecting infectious particles by molecular techniques.

Use of a transfection assay to assess the functional integrity of viral RNA. Due to the large discrepancy between the number of genomic copies detected by real-time RT-PCR and the number of infectious particles detected by a plaque assay, we wanted to assess the integrity of the viral genome in noninfectious MNV-1 samples. To determine whether viral RNA isolated from heat-treated or untreated MNV-1 was still infectious, a transfection assay was set up the following way. Five microliters of viral RNA was mixed with 1 μg of yeast tRNA (Invitrogen) as a stabilizer. RNA was 20 times diluted in Opti-MEMI reduced serum medium (Invitrogen) and transfected into RAW 264.7 cells (1×10^6 cells per 12 wells) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. After a 48-h incubation at 37°C under 5% CO_2, the freeze-thawed lysate was analyzed by a plaque assay to determine the PFU of MNV-1 per milliliter (23). Viral RNA isolated from the unheated MNV-1 lysate resulted in infectious particles after transfection and was detectable by real-time RT-PCR. A similar finding was recently reported for Norwalk virus, where transfection of viral RNA resulted in the generation of viral particles (9). This is in contrast to the findings when RNA was isolated from noninfectious MNV-1 (heated for 150 s at 80°C) and transfected into RAW 264.7 cells. No particles could be recovered despite the detection of the viral genome by real-time RT-PCR. Similarly, no particles were recovered when RNA was isolated from heat-exposed MNV-1 treated with Prot K-RNase A.

In summary, this study demonstrates that exposure to 80°C for 150 s was sufficient to inactivate MNV-1 and shows that real-time RT-PCR did not distinguish between infectious and noninfectious viral genomes. Furthermore, this study shows that heat inactivation had a much stronger detrimental effect on virus infectivity than on the integrity of the viral genome. Because of the major contribution of NoV to viral gastroenteritis infections, quantitative data are urgently needed for the assessment of the public health risks posed by NoV infections. Previous studies have described the use of real-time RT-PCR to screen shellfish (2, 15, 16, 17) and surface water (19) for the presence of human NoV RNA in order to detect NoV contamination. However, the lack of correlation between the presence of genomic copies (detected by real-time RT-PCR) and infectivity (determined by a plaque assay), or even viral genome intactness (determined by a transfection assay), as shown in the present study, should be taken into account in the interpretation of NoV real-time RT-PCR-positive results with relation to the risk to human health, especially when the samples investigated were heat treated.

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