Research Note

Tenacity of Human Norovirus and the Surrogates Feline Calicivirus and Murine Norovirus during Long-Term Storage on Common Nonporous Food Contact Surfaces

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ABSTRACT

The transfer of human norovirus (hNV) to food via contaminated surfaces is highly probable during food production, processing, and preparation. In this study, the tenacity of hNV and its cultivable surrogates feline calicivirus (FCV) and murine norovirus (MNV) on two common nonporous surface materials at two storage temperatures was directly compared. Virus titer reduction on artificially inoculated stainless steel and plastic carriers was monitored for 70 days at room temperature and at 7°C. Viruses were recovered at various time points by elution. Genomes from intact capsids (hNV, FCV, and MNV) were quantified with real-time reverse transcription (RT) PCR, and infectivity (FCV and MNV) was assessed with plaque assay. RNase treatment before RNA extraction was used to eliminate exposed RNA and to assess capsid integrity. No significant differences in titer reduction were found between materials (stainless steel or plastic) with the plaque assay or the real-time quantitative RT-PCR. At room temperature, infectious FCV and MNV were detected for 7 days. Titers of intact hNV, FCV, and MNV capsids dropped gradually and were still detectable after 70 days with a loss of 3 to 4 log units. At 7°C, the viruses were considerably more stable than they were at room temperature. Although only MNV infectivity was unchanged after 70 days, the numbers of intact capsids (hNV, FCV, and MNV) were stable with less than a 1-log reduction. The results indicate that hNV persists on food contact surfaces and seems to remain infective for weeks. MNV appears to be more stable than FCV at 7°C, and thus is the most suitable surrogate for hNV under dry conditions. Although a perfect quantitative correlation between intact capsids and infective particles was not obtained, real-time quantitative RT-PCR provided qualitative data about hNV inactivation characteristics. The results of this comparative study might support future efforts in assessment of foodborne virus risk and food safety.

Human norovirus (hNV), a member of the family *Caliciviridae*, is a nonenveloped virus with a single-stranded RNA genome. This virus is considered the most common causative agent of foodborne outbreaks and sporadic cases of acute nonbacterial gastroenteritis in industrialized countries (26, 33). hNV is transmitted via the fecal-oral route and can be spread directly from human to human via vomitus aerosols or indirectly via contaminated food or surfaces (21). Primary contamination of food can occur by contact with sewage water before harvest; raspberries (15) and strawberries (24) have been reported to be vehicles of hNV. However, the majority of foodborne illnesses are caused by secondary contaminations, which are often a result of hygiene deficiencies associated with food production, processing, and preparation. The virus is easily transferred to contact surfaces (e.g., packaging, benches, sinks, machines, conveyor belts, transport boxes, and latex gloves of food handlers) via contaminated commodities or via fecal matter on the hands of infected handlers and then to the consumed food (11). Epidemiological studies and the fact that hNV is a nonenveloped foodborne virus suggest that hNV has high environmental stability (9). Outbreaks of foodborne illnesses associated with hNV have been reported, with some recurrent incidents, on cruise ships (29, 42), in hotels (10), and on airplanes (39). A few studies have provided experimental evidence for prolonged environmental survival of hNV on nonporous materials that could come into contact with food (12, 14, 22, 23). From a practical point of view, a key question is how long hNV persists on surfaces of machines in food production and processing or on material used for food preparation when introduced into the facility through infected personnel or contaminated products. In this context, more reliable data are needed to evaluate environmental factors such as temperature and storage time and their impact on the stability of hNV on surfaces.

The main problem for hNV inactivation studies is that virus infectivity is not directly measurable. Although various efforts have been made, a functional protocol for cultivating hNV in cell culture is not yet available (13). The “gold standard” in norovirus detection is real-time reverse transcription (RT) PCR, which amplifies specific sequences...
in the isolated viral nucleic acid; however, with this method it is not possible to differentiate between infectious and noninfectious particles (34). To circumvent this problem, genetically related surrogate viruses have been used that are similar to hNV in their resistance to physical and chemical stresses, are easy to cultivate, and offer the possibility of being detected with real-time RT-PCR methods. Norovirus persistence on nonporous surface materials has been investigated with feline calicivirus (FCV) and murine norovirus (MNV) (12, 16, 37), both of which are non-enveloped viruses belonging to the family *Caliciviridae*. Results from studies in which direct comparisons of FCV and MNV were carried out indicate differences in stability under various physical conditions and in susceptibility to environmental factors (8, 18, 35). Controversy remains as to whether and to what extent results from inactivation studies with the surrogates FCV and MNV are transferable to hNV. Tulane virus was recently suggested as a surrogate for hNV because of its ability to bind to the same host cell receptors (18). Apart from a closer phylogenetic relationship to hNV, MNV is likely a better surrogate than Tulane virus because of its persistence over a wider range of pH values, at 2 ppm of chlorine, and without a loss of titer at 4°C (17). However, more comparable data are needed about the tenacity of hNV and its surrogates on various food contact surfaces and under different storage conditions commonly used for maintaining food safety (e.g., cool storage).

In this study, the persistence of hNV and the cultivable surrogates FCV and MNV on relevant surfaces was directly compared. Comparative data were generated with various quantitative methods to determine the presence of intact capsids (real-time RT-PCR) and number of infective particles (plaque assay). The aim of the study was to investigate differences in virus titer reduction associated with (i) storage on plastic and stainless steel and (ii) storage at room and refrigeration temperatures. The goal was also to determine which surrogate for hNV is the more appropriate in this context and whether real-time quantitative (q) RT-PCR for hNV provides reliable data in tenacity studies. To our knowledge, the approach to directly compare hNV and both surrogates by genome detection (hNV, FCV, and MNV) and infectivity assessment (FCV and MNV) is unique. These results should provide more comprehensive insight into norovirus persistence and support future efforts in foodborne virus risk assessment and food safety.

**MATERIALS AND METHODS**

**Preparation of virus stocks and virus propagation in cells lines.** hNV (GIL.3) stock was obtained from a stool sample, which was diluted 1:5 with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$; pH 7.4) and clarified by centrifugation at 3,000 × g for 15 min. The supernatant was mixed with 15% glycerol in PBS (final glycerol concentration of 10%) and filtered through a 0.45-μm-pore-size polyethersulfone syringe filter (VWR International GmbH, Darmstadt, Germany). FCV isolate KS20 (kindly provided by the Robert-Koch-Institute, Berlin, Germany) and MNV isolate S99 (kindly provided by the Institute of Animal Hygiene and Veterinary Public Health, University of Leipzig, Leipzig, Germany) were propagated in confluent monolayers of Crandall Reese feline kidney cells and murine macrophage cell line RAW 264.7 (ATCC TIB-71) as described by Bidawid et al. (7) and Wobus et al. (43), respectively. Viral cell cultures were subsequently subjected to two freeze-thaw cycles (−150°C for 30 min) to facilitate liberation of virions from infected cells. All virus stocks were stored in aliquots at −150°C until used.

**Surface materials.** Brushed stainless steel and plastic (polyethylene) carriers (20 mm in diameter) were cleaned in accordance with the requirements of DIN EN 13697 (3).

**Experimental workflow.** A 50-μl volume of each virus inoculum was pipetted in the center of a carrier surface and incubated in an evacuated desiccator at 450 mbar vacuum for approximately 60 min until the suspension was visibly dried. Virus-inoculated carriers were stored in sealed plates protected from light under ambient relative humidity at room temperature (21 ± 5°C, 54 to 69% relative humidity [RH]) and in the refrigerator at 7 ± 1°C (49 to 52% RH). At various points during storage (6 h, 1, 7, 14, 21, and 70 days at room temperature and 7, 14, 21, and 70 days at 7°C), residual viruses were recovered from the carrier by rinsing with 2 ml of culture medium. As a reference value for the calculation of virus titer reduction, particles recovered from the carrier at the beginning of an experiment (time point zero) were used. The average reference virus titers were 3 × 10$^{6}$ particles per ml for hNV, 2 × 10$^{5}$ particles per ml for FCV, and 1 × 10$^{5}$ particles per ml for MNV. The amount of FCV and MNV in 500 μl of each sample suspension was titrated in cell lines with plaque assays. From the same batch, 140 μl was used for quantification of hNV, FCV, and MNV with real-time qRT-PCR. Before template nucleic acid extraction, exposed RNA molecules were digested by adding 35 μg of RNase A (QIAGEN GmbH, Hilden, Germany) per sample and incubating the mixture for 1 h at 37°C. After digestion, the RNase activity was inhibited by adding 140 U of RNase inhibitor (QIAGEN GmbH) per sample and incubating the mixture for 30 min at room temperature. With the RNase pretreatment, detection of unexposed RNA molecules and genomes from structural intact capsids was performed to assess capsid integrity and virus infectivity by molecular methods. Each time point analysis (including a control consisting of virus stock in suspension) was conducted with at least two replicates (carriers) in independent experiments and on different days.

**Plaque assays for quantification of infectious FCV and MNV.** The infectivity of FCV and MNV was determined by plaque assay as described by Bidawid et al. (7) and Wobus et al. (43), respectively. Virus titers were calculated as the weighted average number of PFU per milliliter according to the standard DIN EN 14476 (4).

**Nucleic acid extraction.** Virus RNA was extracted by the column centrifugation method using the QIAamp viral RNA mini kit (QIAGEN GmbH) according to the manufacturer’s protocol. To avoid residual RNase activity, 50 U per sample of Protector RNase inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) was added, and the mixture was incubated for 30 min at room temperature. The nucleic acid was immediately used in a downstream PCR application or stored at −19°C until used.

**Primer and probe design.** Primer and probe sequences for FCV and MNV detection were selected based on a consensus sequence resulting from a multiple sequence alignment calculated using CLUSTAL W (38). Virus nucleotide contigs were obtained from nucleotide databases (GenBank, RefSeq Nucleotides, EMBL,
and DDBJ). The oligonucleotides were designed using CLC Combined Workspace (v. 3.01, CLC bio, Aarhus, Denmark) and purchased from Eurofins MWG Operon (Ebersberg, Germany). For detection of FCV, primers and probe were used as previously described (2). The FCV probe was modified with a black hole quencher 1 (BHQ1). MNV detection was performed with primers MNV_fwd_HS-OWL (GAA TGA GGA TGA GTG ATG G), MNV_rev_HS-OWL (CAA TTT GGT TNA TTT GCC CG), and MNV_probe_HS-OWL ([FAM]-GAT CTT GTT CCT NCC GCC-[BHQ1]). hNV primers and probe were used as described previously (30).

Real-time RT-PCR for detection of hNV, FCV, and MNV. Real-time RT-PCRs were performed as one-step monoplex assays with hydrolysis probes (TaqMan) using the QuantiTect Virus + ROX vial kit (QIAGEN GmbH). Each PCR mixture (final volume of 15 μl) consisted of 5 μl of QuantiTect Virus NR Master Mix, 0.25 μl of QuantiTect Virus RT-Mix, 500 nM final concentrations of each primer, 250 nM final concentrations of a probe, and nuclease-free water. The final volume of each reaction mixture was adjusted to 25 μl by adding 10 μl of RNA solution (template) or RNase-free water (PCR negative control). Amplification and detection were carried out with the Rotor-Gene 3000 (Corbett Research, Sydney, Australia). The thermal cycling conditions consisted of reverse transcription at 50°C (30 min for hNV; 20 min for FCV and MNV), activation at 95°C (5 min for hNV; 10 min for FCV and MNV), and amplification for 50 cycles with denaturation at 95°C for 15 s and annealing and extension at 60°C (45 s for hNV; 55 s for FCV and MNV).

Quantification of hNV, FCV, and MNV with real-time RT-PCR. Quantification of hNV, FCV, and MNV with real-time RT-PCR was performed by using standard curves that describe the relationship between a given cycle threshold value and the corresponding number of genome equivalents per milliliter. Quantification of hNV was performed as previously described (30). Recombinant RNA standards and external standard curves for FCV and MNV quantification were constructed as described elsewhere (25, 30). Serial dilutions (10-fold) of the purified recombinant standards were used as a template in the real-time qRT-PCR assays as described. External standard curves were generated by plotting the cycle threshold values for each dilution against the respective number of RNA copies per milliliter. FCV and MNV quantities were expressed as genome equivalents.

Calculation of log virus titer reduction and data analysis. Virus reduction is presented as the average log reduction and was calculated as the difference between the virus titer measured at a defined storage time and the reference virus titer at time zero, which was used as the baseline for each experimental replicate. Diagrams and statistics (e.g., linear regression line, coefficient of determination, slope, and standard deviation) were generated using Microsoft Excel (Microsoft Corp., Redmond, WA).

RESULTS AND DISCUSSION

Environmental stability of hNV, FCV, and MNV on different surface materials. The comparison of the data sets obtained for virus persistence on stainless steel and plastic, both of which are food contact materials, revealed no significant differences in infectivity as determined with the plaque assay (FCV and MNV) or in the number of intact capsids as determined with real-time qRT-PCR (hNV, FCV, and MNV). Figure 1 shows the logarithmic reduction of FCV on stainless steel and plastic carriers determined at different time points (6 h and 1, 7, 14, 21, and 70 days) with the real-time qRT-PCR. In previous studies, no differences in virus reduction were observed on different carrier materials for hNV and MNV (stainless steel, ceramic tile, and formica) (14) and for FCV (stainless steel, ceramic, and formica) (12). When contaminated, such nonporous surfaces might not support the reduction of these highly infectious noroviruses and therefore facilitate their spread. In contrast, copper alloys has antimicrobial activity against noroviruses. In a recent study, rapid inactivation of MNV occurred at room temperature on alloys containing more than 60% copper but no inactivation was noted on dry stainless steel surfaces (41).

Impact of storage temperature on the tenacity of hNV, FCV, and MNV on stainless steel. The results from persistence studies at ambient (room temperature) and refrigeration (7°C) temperatures are shown in Figure 2. The inactivation profiles of hNV, FCV, and MNV are shown for storage on stainless steel carriers. Both the type of material and environmental factors such as RH affect the stability of foodborne viruses on surfaces (1, 19). Therefore, storage of virus-contaminated material was conducted under constant environmental conditions to limit interfering factors and to mimic realistic conditions found at food handling locations. To ensure direct comparability, quantification of infectivity (FCV and MNV) and documentation of intact capsids (hNV, FCV, and MNV) were performed on samples from the same virus batch.

At room temperature the infectivity of FCV and MNV (Fig. 2A) decreased within 6 h and 1 day, respectively, by 1 to 3 log units. At 7 days postinoculation, the titers of both viruses were reduced below the detection limit of 4.5 log units. D’Souza et al. (12) and Cannon et al. (8) observed a 4- to 5-log reduction in infective FCV and infective FCV and MNV, respectively, after 5 to 7 days on stainless steel. These findings clearly indicate a rapid reduction in number
of infective particles during storage under dry conditions at room temperature.

At a storage temperature of 7 °C, the reduction in number of infectious particles was remarkably slower than that at room temperature (Fig. 2B). For FCV, a 2-log reduction in titer was noted after 7 days and a 3-log reduction was noted after 70 days on steel surfaces. In contrast to FCV, MNV titer after 70 days of storage was reduced by less than 1 log unit. Cannon et al. (8) reported that in suspension MNV was more stable than FCV at room temperature and refrigerated MNV was only slightly more stable than FCV. This finding contrasts with the findings in our study, which might be a result of differences in experimental design and methodology. Nevertheless, both studies clearly demonstrated that FCV and MNV differ in stability, and MNV might be a more stable surrogate for hNV in this context. Our observations that infectious FCV and MNV are significantly more stable when stored under refrigerated conditions are consistent with the results of other studies (8, 12, 14).

A rapid reduction in virus titer (hNV, FCV, and MNV) at room temperature also was found with the real-time qRT-PCR assay (Fig. 2C). The number of intact capsids decreased by approximately 1 log unit within 6 h to 1 day. Subsequently, the virus titers gradually dropped by 1 to 2 log units between 7 and 21 days and by approximately 3 log units by 70 days.

Under cool storage conditions (7 °C), no significant reduction of intact capsids was noted for hNV, FCV, and MNV (Fig. 2D). These titer reductions of <1 log unit indicate that the viruses were stable over the entire 70-day test period. Although plaque assays revealed that infective MNV was more stable than FCV at 7 °C (Fig. 2B), the reduction profiles of all three test viruses measured with real-time qRT-PCR were nearly identical at both storage temperatures.

Results obtained with both the plaque assays and the real-time qRT-PCR clearly indicate that hNV and its surrogates FCV and MNV inoculated on nonporous surfaces are much more stable under cool storage conditions than at room temperature. Therefore, cool storage as usually conducted by the food industry and the consumer to prevent bacterial growth may be counterproductive in the case of noroviruses for ensuring food safety. The reduction profiles obtained in this study with an initial drop in virus titer within a short time followed by stable titers and further inactivation until the end of the experimental period seem to be characteristic for hNV, MNV, and FCV. Similar reduction characteristics were observed for FCV on stainless steel, formica, and ceramic (12) and on metal discs (27) and for MNV on stainless steel (37).

Results from studies with MNV (16) and human enteric viruses (1, 28) on food and environmental materials suggest that moisture might be the key factor for virus inactivation.
on surfaces. Although studies on a molecular level have not been conducted, the loss of moisture seemed to play a dominant role in virus inactivation in this study. Takahashi et al. (37) concluded that food residues exert a protective effect against desiccation of noroviruses based on the observation that MNV is significantly more stable during storage on stainless steel with defined food residues (cabbage, ground pork, and lettuce) than on surfaces without an organic load. Escudero et al. (14) also suspected that moisture and likely food components play an important role in virus stability.

**Usability of real-time RT-PCR for quantification of infectious hNV in tenacity studies.** Real-time qRT-PCR quantifies viral genome copies by detecting nucleic acid that could originate from infectious viruses, noninfectious but intact capsids, or free RNA molecules released from disrupted particles even when partially degraded. Such released RNA lead to false-positive PCR results and therefore to an overestimation of intact capsids and putative infectious particles. In some inactivation studies, the amount of viral RNA detected was not correlated with infectious virus (5, 6, 32). Approaches with integrated RNase pretreatment were devised to overcome that problem and detect primarily genomes from structurally intact capsids (31, 40), although RNA from disassembled capsids might still be protected by capsid proteins and thus produce a PCR signal. Quantification of intact virus particles with real-time qRT-PCR provides data on the stability of RNA viruses when cell culture-based infectivity assays are not efficient or not available (30, 36). Whether the number of intact particles allows solid statements about the level of infectious viruses in general and especially in the context of tenacity studies is still unclear (20). The approach used in this study was to correlate real-time qRT-PCR and plaque assay data from both of the surrogates FCV and MNV and to directly compare the PCR data of the surrogates and the target pathogen hNV. The results of our study indicate that although the reduction profiles of FCV and MNV at room temperature tend to be identical, the results of the two detection methods are not correlated quantitatively. The number of infectious viruses (Fig. 2A) was reduced more than the number of intact capsids (Fig. 2C). Direct comparison of hNV, FCV, and MNV inactivation at room temperature (Fig. 2C) revealed almost identical reduction profiles. At cool storage, both methods consistently revealed no reduction in MNV titer. These results confirm that real-time qRT-PCR is not an appropriate method for determination of the level of infectious viruses but provides relative quantification data for monitoring reduction profiles and therefore the persistence of hNV on nonporous surfaces. Until an in vitro model for the evaluation of hNV infectivity is available, real-time qRT-PCR represents the method of choice for virus detection and quantification. Because epidemiological surveillance studies associated with hNV outbreaks have corroborated long-term infectivity on surfaces, the detection of viral genomes might overestimate but still represent an adequate indicator for potential infectious hNV particles.

This comparative study meets the need for more information concerning the relevance of the cultivable surrogates FCV and MNV and provides data for a more comprehensive view of hNV persistence on food contact surfaces. The results suggest that hNV remains infective for prolonged periods that are relevant for the food industry and the consumer. Contaminated surfaces thus serve as a transmission vehicle for infective virus, which represents a risk for human health. Consequently, to improve product safety, appropriate disinfection measures for effective inactivation of hNV on such surfaces used for food production, processing, or preparation are needed. MNV appears to be more stable than FCV when stored at low temperatures and thus is a more suitable hNV surrogate for virus tenacity studies. FCV is a respiratory virus and is more sensitive to low pH conditions (compared with MNV) (8); thus, MNV might be a more promising hNV surrogate for inactivation studies in general. The data obtained in this study may be useful in the development of risk assessment systems for hNV, surface decontamination strategies, and food handling practices to improve consumer protection in the food sector and domestic environments.

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