

Research Note

Attachment of Noroviruses to Stainless Steel and Their Inactivation, Using Household Disinfectants

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ABSTRACT

The aims of this study were (i) to evaluate the impact of pH and relative humidity on the attachment of norovirus (NoV) to fomites and (ii) to evaluate the effectiveness of different household disinfectants on NoV attached to fomites. Plaque assay and/or real-time reverse transcription PCR assay were used to determine the amount of murine and human NoV attached to stainless steel disks, i.e., the amount removed by sonication in elution buffer but not by surface rinses with water only. An enzymatic pretreatment was used for both human and murine NoV before the real-time reverse transcription PCR assay to avoid detection of RNA associated with inactivated virus. For both murine and human NoV, maximum attachment was obtained after a contact time of 10 min. Attachment of NoV to stainless steel does not appear to be affected by pH, although murine NoV was less attached (<2 log units) at pH 9 and at low relative humidity (25%) than was human NoV (3 log units). Sodium hypochlorite (3%) was the most effective disinfectant, producing a greater than 3-log reduction after 10 min compared with less than a 1-log reduction after treatment with quaternary ammonium compounds and ethoxylated alcohols. Murine NoV was more sensitive than human NoV to disinfectants by approximately 1 to 2 log units. These results will help improve strategies for decontaminating surfaces harboring NoV and thus reduce the incidence of illness caused by these pathogens in the food sector and domestic environments.

Enteric viruses are transmitted via the fecal-oral route, and contaminated individuals, water, food, and environmental fomites are the main vehicles. Nevertheless, there is a lack of information on the mechanism of transmission via fomites. In 2009, Vugia et al. (23) reported that foodborne infections in the United States have not been significantly reduced by strict sanitary measures. Although disinfection of food contact surfaces is believed to be effective for stopping the transmission of norovirus (NoV), human NoV cannot be cultured *in vitro*, hampering studies of viral inactivation (7). Outbreak investigations indicate that the human NoV is relatively resistant to common disinfectants (5, 20). Cultivable surrogates such as feline calicivirus (FCV) have been used as models to evaluate the persistence and inactivation of NoV. Murine norovirus (MNV) was isolated in 2003 (8) and is now considered the most representative surrogate for human NoV (24). A recent report comparing MNV and FCV revealed that MNV was more acid tolerant than FCV, supporting MNV as a more suitable surrogate for human NoV (4). MNV was therefore chosen for comparison with human NoV in the present study.

NoV spread is also impacted by such parameters as pH, relative humidity, temperature, and surface type, which

affect viral attachment to fomites. Other researchers have examined the attachment of FCV (6), poliovirus (1), hepatitis A virus (1, 14, 15), and rotavirus (1) to surfaces. All enteric viruses survive better at 4°C than at 20°C, with the exception of rotavirus (1). High relative humidity is favorable to enteric virus survival (1), although Mbithi et al. (15) found that hepatitis A virus persists longer at low relative humidity. Cannon et al. (4) found that MNV was resistant to pH changes, organic solvents, and various environmental conditions. However, no studies have quantified the ability of noroviruses to attach to surfaces under different conditions. The aims of this study were to evaluate the effect of environmental factors on the attachment of NoV to fomites and the efficacy of common household chemical disinfectants for inactivating the attached virus.

MATERIALS AND METHODS

NoVs. Human NoV (BC 728, GenBank accession no. FJ573213) was obtained from the British Columbia Centre for Disease Control (Vancouver, British Columbia, Canada) in 2007. MNV strain 1 was propagated in RAW 264.7 cells as described previously by Wobus et al. (24). The titer of the cell lysate stock solution was estimated at 1×10^7 PFU/ml.

Plaque assay. The plaque assay was carried out as described by Macinga et al. (13) except that 0.1% crystal violet was used for cell staining (21).

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TABLE 1. Disinfectants and neutralizers used in this study

Product	Active ingredient	pH
Disinfectant 1	3% Sodium hypochlorite when packed	12.35
Disinfectant 2	2-(1-Butoxy) propanol and ethoxylated alcohols	9.79
Disinfectant 3	0.08% <i>N</i> -alkyl dimethyl benzyl ammonium chloride (1% C ₈ , 1% C ₁₀ , 67% C ₁₂ , 25% C ₁₄ , 7% C ₁₆ , 1% C ₁₈); 0.02% <i>N</i> -alkyl dimethyl benzyl ammonium chloride (40% C ₁₂ , 50% C ₁₄ , 10% C ₁₆)	10.66
Neutralizer 1	4% Lecithin and 28% Tween 80	6.14
Neutralizer 2	1% Sodium thiosulfate (Na ₂ S ₂ O ₃)	5.98

RNA extraction for RT-PCR assay. Nucleic acid extraction for the reverse transcription (RT) PCR assay was carried out using the magnetic extraction kit according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France). RNA extracts were stored at -80°C until use.

Amplification of NoV RNA by real-time RT-PCR. The enzymatic pretreatment described by Lamhoujeb et al. (10) was applied to all viral samples to eliminate signal associated with inactivated virus. The following primers and probe were designed for MNV amplification and detection: GCTGCGCCTCTC-TTGAC (MGmnv-1Forward), AGGGATGGTGTCTGAAAACC (MGmnv-1Reverse), and 6FAM-TTCGTGCGGTCCCAAGATC-CATCT-TAMRA (MNV-1Probe). The amplification of MNV and human NoV GII was performed as described by Ngazoa et al. (17)

using TaqMan one-step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA) on an ABI 7500 real-time PCR platform (Applied Biosystems). For MNV, the annealing and extension temperature was changed to 58°C. Negative controls were composed of 5 µl of sterile diethylpyrocarbonate water.

NoV attachment to stainless steel. The attachment of human NoV and MNV to stainless steel disks at different pH and 25% (±3%) or 80% (±5%) relative humidity (RH) was tested using virus suspended in 0.1 M acetate (pH 4), phosphate (pH 7), and carbonate (pH 9) buffers. A different desiccation vessel (20-cm diameter and 21 cm high) was used for each contact time (10 min, 1 h, and 2 h) to maintain precise humidity (12). Each sterile disk was placed in a 12-well microtiter plate in a vessel and contaminated by spotting 10 µl of MNV suspension diluted to

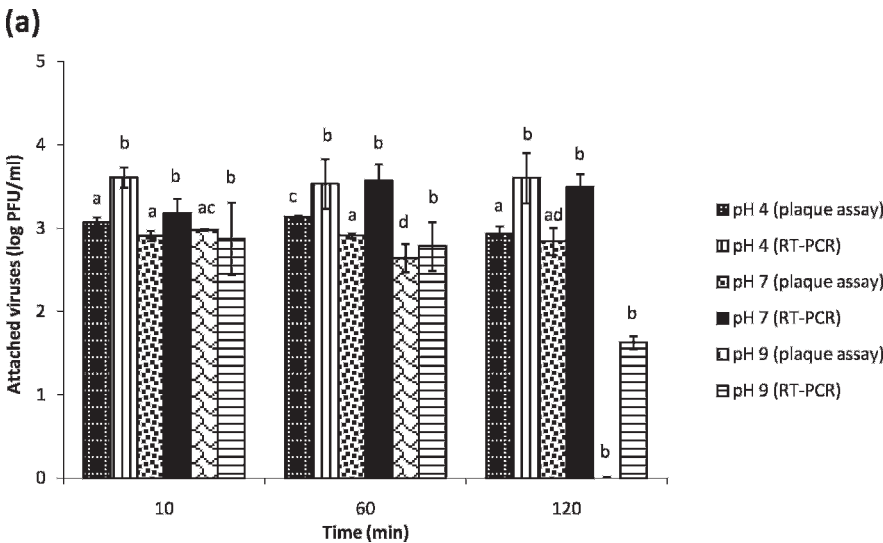


FIGURE 1. Attachment of MNV to stainless steel at pH 4, 7, and 9 and 25% (a) or 80% (b) relative humidity. Results are expressed as log PFU per milliliter for attached viruses as a function of contact time (minutes) using the plaque assay and the real-time detection systems. Treatment results with different letters are significantly different at P < 0.05.

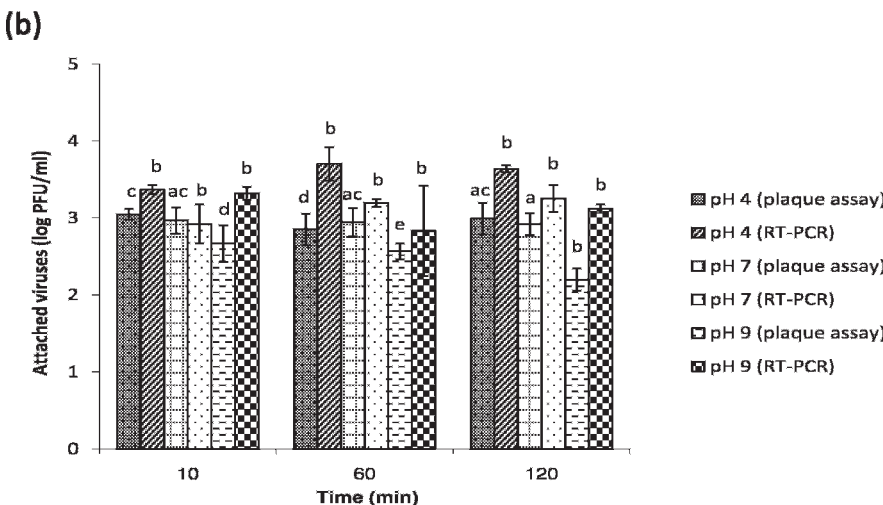
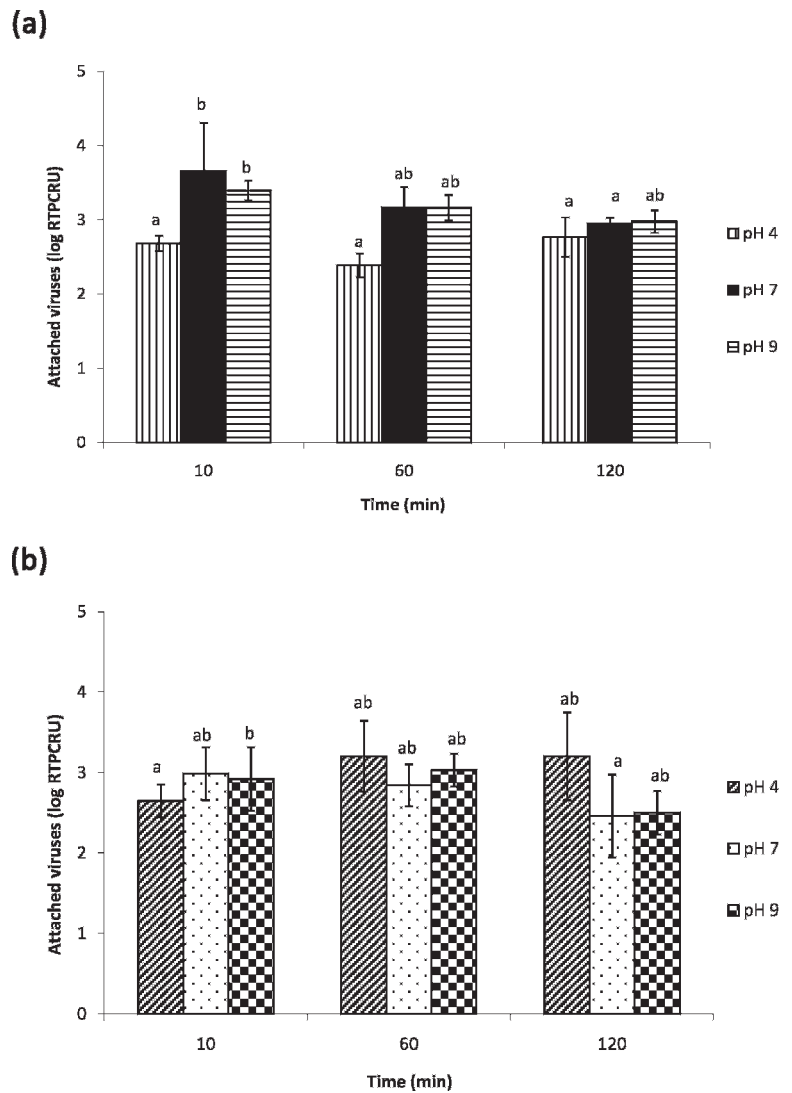


FIGURE 2. Attachment of human NoV to stainless steel at pH 4, 7, and 9 and 25% (a) or 80% (b) relative humidity (log RTPCRU based on real-time RT-PCR assay). Treatments results with different letters are significantly different at $P < 0.05$.



10^6 PFU/ml (i.e., about 10^4 PFU) or 20 μ l of a 10% solution of human NoV GII (i.e., about 10^4 RT-PCR units [RTPCRU]) in the appropriate buffer. The disk was then rinsed once by pipetting 250 μ l of deionized water over the surface, and the remaining attached virus was recovered by adding 500 μ l of Earle's balanced salt solution (EBSS) in 20% tryptose phosphate broth (TPB), vortexing for 45 s, and sonicating (ultrasonic cleaner 200, Branson, Danbury, CT) for 10 min. Half of the sample was used for the infectivity assay, and the rest was used for molecular detection.

Chemical inactivation of NoV attached to stainless steel.

Household disinfectants and neutralizers used in this study are presented in Table 1. Sterile (6) stainless steel disks (1 cm in diameter) were placed in a 12-well microtiter plate, contaminated by spotting 10 μ l of undiluted MNV suspension (approximately 10^5 PFU) or 20 μ l of human NoV suspension (10^4 RTPCRU), and allowed to dry for 40 min in a laminar flow hood. Twenty microliters of disinfectant was then placed on the same spot for 5 or 10 min at room temperature. The disk was placed in a tube containing 980 μ l of EBSS–20% TPB, the appropriate neutralizer was added, and the pH was adjusted to 7.0 with HCl. The viruses were recovered by sonication as described above and subjected to the appropriate detection procedures.

Statistical analysis. Infectious virus and viral genome (RNA) number were expressed as log PFU per milliliter and log RT-PCR

units, respectively. Log reduction of RNA was based on real-time RT-PCR threshold cycle (C_t) and the slope of C_t versus the log number. Statistical analyses were performed with STATGRAPHICS Plus 4.1 (Manugistics Inc., Rockville, MD). Significant differences among mean values were tested by analysis of variance. Treatment comparisons were performed using Fisher's least significant difference test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Detection of NoV by real-time RT-PCR. The new real-time RT-PCR method developed in this study for MNV produced highly correlated results for the threshold cycle and the viral content of the samples ($y = -3.0416x + 28.154$; $R^2 = 0.9854$). The specificity was confirmed using heterologous RNA from rotaviruses (Wa), human NoV (GII.4 and GI.x), FCV, and hepatitis A virus (data not shown). A standard curve also was established for human NoV ($y = -3.1906x + 29.811$; $R^2 = 0.9949$) using the TaqMan RT-PCR as previously described (17).

Attachment of NoV to stainless steel. Figure 1 shows the attachment of MNV to stainless steel disks, expressed as log PFU in plaque assays. The maximum attachment of 10^3 PFU was obtained after a contact time of 10 min. Extending

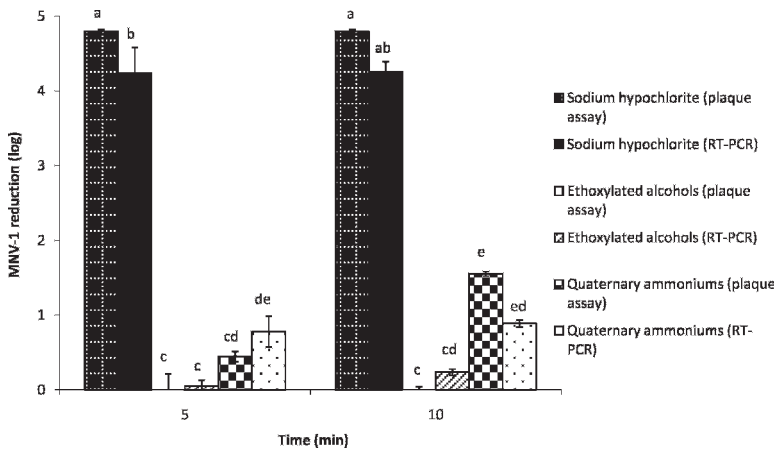


FIGURE 3. Inactivation of MNV on stainless steel after exposure to chemical disinfectants (log reduction based on plaque assay and real-time RT-PCR assay after 5 or 10 min of contact). Treatment results with different letters are significantly different at $P < 0.05$.

the contact time to 60 or 120 min did not increase attachment. At both RH values, the pH did not appear to have any effect on MNV attachment, except for pH 9, where the virus may have been inactivated after 120 min of incubation. This decrease in titer was more significant at low RH. Similar results were obtained with human NoV (Fig. 2).

Our results are in agreement with those of Abad et al. (1), who reported that nonenveloped viruses survive better at high (>80%) RH. Different results were reported by Mbithi et al. (15), who reported that hepatitis A virus and rotavirus did not survive well at high RH. In water, FCV was less stable than MNV and human NoV GI (3). According to Olson et al. (18), low pH can damage viral capsid proteins or exposed nucleic acid. A pH near or below the isoelectric point may result in a significant loss of viral infectivity, perhaps due to aggregation (9, 11). In our experiments, the MNV titer decreased after 2 h at basic pH and low RH. Similar results were reported by Stine et al. (22) for *Escherichia coli* ATCC 25922 and FCV.

Chemical inactivation of NoV attached to stainless steel. Neutralizing sodium hypochlorite with sodium thiosulfate or quaternary ammonium disinfectant with lecithin allowed determination of viral titer by plaque assay. For the experiments, neutralizer 1 was used with

disinfectants 2 and 3, and neutralizer 2 was used with disinfectant 1.

Figure 3 shows the sensitivity of MNV on stainless steel to the different chemical disinfectants. Total inactivation was observed with sodium hypochlorite contact times of 5 and 10 min using the plaque assay, whereas 4-log reductions were obtained with the TaqMan RT-PCR assay in combination with enzymatic pretreatment. For ethoxylated alcohols, no significant reduction was observed with either detection method. An approximately 1-log reduction after 10 min and a less than 0.5-log reduction after 5 min were observed for quaternary ammonium compounds, with either the plaque assay or the TaqMan RT-PCR assay.

Figure 4 shows the results obtained for human NoV. Total inactivation was obtained with the sodium hypochlorite-based disinfectant after a contact time of 10 min; however, only a 2-log reduction was obtained after 5 min. No reduction was observed when either ethoxylated alcohols or quaternary ammonium compounds were applied.

Few studies on the inactivation of enteric viruses of the family *Caliciviridae* have been published, and these studies frequently include assessment of the sensitivity of viruses in solution. In most of these studies, NoV surrogates such as FCV and MNV were used because human NoVs do not replicate in vitro (7, 24). Surrogates often are compared with each other, but the differences between the surrogates and

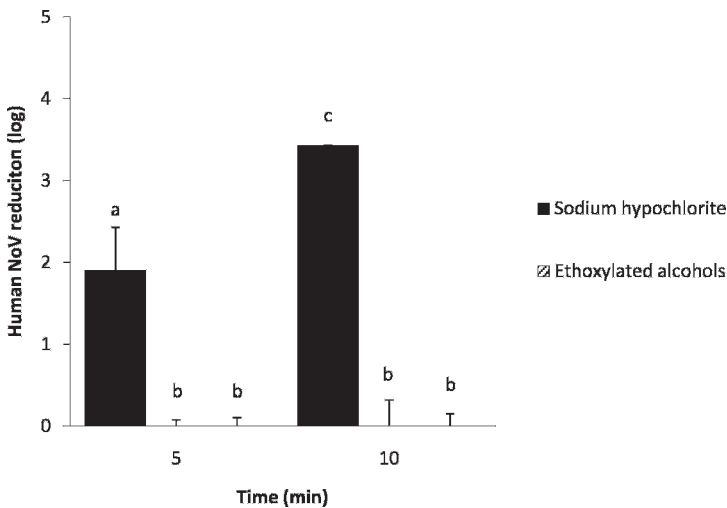


FIGURE 4. Inactivation of human NoV on stainless steel after exposure to chemical disinfectants (log reduction based on real-time RT-PCR assay after 5 or 10 min of contact). Treatment results with different letters are significantly different at $P < 0.05$.

human NoV have not been well characterized. In this work, both human NoV and MNV were used to evaluate the sensitivity of NoV to household chemical disinfectants. Our study revealed that MNV was more sensitive than human NoV to chemical disinfectants.

In the evaluation of disinfection efficacy, only sodium hypochlorite was effective against NoV, as determined by cell culture and real-time RT-PCR assay. The observed inactivation exceeded 4 log units for MNV and 3 log units for human NoV. According to the Health Canada definition of a disinfectant (2), only products containing sodium hypochlorite should be considered effective against NoV. Our results are in agreement with previously published findings indicating that sodium hypochlorite-based products are the chemical agents most effective against NoV (3, 19). We also have confirmed that alcohols and ammonium-based disinfectants are less effective against nonenveloped viruses (16, 21). This lack of efficacy is a concern because 38% of disinfectants used in the United States for hard surfaces are quaternary ammonium compounds (21). In this study, we also found that results obtained with real-time RT-PCR assays are not always consistent with those obtained by plaque assays. This discrepancy is likely due to the limitation of molecular methods, which detect viral RNA independently of viral viability and infectivity. The real-time RT-PCR assay developed in our laboratory for MNV seems to be a viable alternative to plaque assays; it is sensitive, specific, and faster and generates reproducible results.

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