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Laboratory Evidence of Norwalk Virus Contamination on the Hands of Infected Individuals

Pengbo Liu,a Blanca Escudero,b Lee-Ann Jaykus,b Julia Montes,a Rebecca M. Goulter,b Meredith Lichtenstein,a Marina Fernandez,a Joong-Chul Lee,a Elizabeth De Nardo,a Amy Kirby,a James W. Arbogast,c Christine L. Moea Center for Global Safe Water, Hubert Department of Global Health, Emory University, Atlanta, Georgia, USA; Food Bioprocessing and Nutrition Sciences, North Carolina State University, Raleigh, North Carolina, USA; Gojo Industries Inc., Akron, Ohio, USA

Human norovirus (NoV) outbreak investigations suggest that the hands of infected individuals play an important role in NoV transmission. However, there is no experimental evidence documenting the likelihood and degree of NoV contamination on hands. As part of a clinical trial designed to evaluate the efficacy of high-pressure processing for Norwalk virus (NV) inactivation in oysters, 159 hand rinse samples were collected from 6 infected and 6 uninfected subjects. NV was concentrated from the samples by polyethylene glycol precipitation, followed by RNA extraction using an automated guanidinium isothiocyanate-silica method. NV RNA was detected and quantified using multiple NV-specific reverse transcription-quantitative PCR (RT-qPCR) assays. A total of 25.4% (18/71) of the hand rinse samples collected from 6 infected volunteers were presumptively positive for NV, with an average of 3.86 log10 genomic equivalent copies (GEC) per hand. Dot blot hybridization of PCR products obtained using a different primer set, and DNA sequencing of selected amplicons, provided further confirmation of the presence of NV in the hand rinses. NV contamination was also detected in two hand rinse samples obtained from one uninfected subject. These findings provide definitive evidence of NV contamination on the hands of infected subjects observed under controlled clinical research conditions. Such data support the need for better hand hygiene strategies to prevent NoV transmission.
Determination of volunteer infection status. The infection status of volunteers was defined as (i) reverse transcription-PCR (RT-PCR) detection of NV RNA in any postchallenge stool (16) or vomitus sample(s) and (ii) seroconversion as the detection of NV RNA in hand rinse samples. Seroconversion was defined as an enzyme-linked immunosorbent assay (ELISA) with Norwalk virus-like particles (18).

Viral concentration and RNA extraction. The viruses in 25-ml volumes of each hand rinse sample were precipitated by 12% polyethylene glycol (PEG; molecular weight of 7,000 to 9,000) after samples were adjusted to 0.9 M NaCl and supplemented with 1% bovine serum albumin (Sigma-Aldrich). After incubation for 2 h at 4°C, samples were spun at 10,000 × g for 20 min, and the precipitated pellet was suspended in 1 ml of 1× phosphate-buffered saline (PBS; MP Biomedicals, Solon, OH). Extraction of NV RNA was performed on the entire 1-ml concentrate using a NucliSENS easyMAG guanidinium isothiocyanate-silica automated system (bioMérieux SA, Marcy l’Étoile, France) according to the manufacturer’s instructions, with a final elution volume of 50 μl.

Detection of NV in hand rinse samples by RT-qPCR. To monitor PCR inhibition, a homologous internal amplification control (IAC) was generated in accordance with the method of Abdulmawjood et al. (19). This IAC could be amplified using the NV diagnostic primers but had an internal sequence corresponding to a region of the pUC19 vector (New England BioLabs, Ipswich, MA). To account for the possibility of outcompeting the IAC when target template levels were low, NV RNA extracted from hand rinse samples was amplified by RT-qPCR both with and without coamplification of the IAC (total of 17 samples). When the IAC was not detected, samples were diluted 1:4 and/or 1:10 and reamplified. Those samples that consistently produced Ct values ≥ 38 in two duplicate amplifications with IAC coamplification were considered presumptively positive.

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Confirmation of positive samples by RT-PCR, hybridization, and sequencing. Positive samples detected by the aforementioned RT-qPCR method were further confirmed by a conventional RT-PCR amplification using a COG1F/COG1R primer set (21) (Table 2), DNA hybridization, and, in some instances, cloning and sequencing. For confirmation, dot blot hybridization of amplicons was performed using a digoxigenin (DIG) nucleic acid detection kit (Roche Diagnostics Corp., Indianapolis, IN) according to the manufacturer’s instructions. After hybridization, the membrane was washed and then processed using a DIG wash block buffer set (Roche) and DIG nucleic acid detection kit (Roche). A positive result was identified by a blue precipitate.

For further confirmation, a subset of the conventional RT-PCR amplicons corresponding to hybridization-confirmed NV-positive

### TABLE 1 Volunteer infection status and number/type of hand rinse samples collected post-NV challenge

<table>
<thead>
<tr>
<th>Volunteer status</th>
<th>Subject ID</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>34</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>36</td>
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<td>2</td>
<td>2</td>
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<td></td>
<td>40</td>
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<td>2</td>
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<tr>
<td></td>
<td>46</td>
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<td>3</td>
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<td>54</td>
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<td></td>
<td>Total</td>
<td>38</td>
<td>2</td>
<td>4</td>
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<td>44</td>
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<td>55</td>
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<tr>
<td>Total</td>
<td>71</td>
<td>4</td>
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<table>
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<tr>
<th>Volunteer status</th>
<th>Subject ID</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
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</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Day 0** Bath Vital N/A Bath Vital N/A Bath Vital N/A
- **Day 3** Bath Vital N/A Bath Vital N/A Bath Vital N/A
- **Day 4** Bath Vital N/A Bath Vital N/A Bath Vital N/A

**Volunteer status**:
- Infected
- Uninfected

**Subject ID**:
- 34
- 36
- 37
- 40
- 46
- 54
- 38
- 41
- 42
- 44
- 47
- 55

**Total**:
- 71
- 88

**ID**, identification number. Bath samples were collected after defecation. Vital sign samples were collected during vital sign measurement. N/A, sample collection time not clear.
hand rinse samples with relatively low \( C_T \) values (<30) were purified using a QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) and cloned into TOPO vector using a TOPO cloning kit (Invitrogen, Carlsbad, CA). The DNA insertion in each clone was sequenced by Genewiz Inc. (Research Triangle Park, NC) using the universal M13R and M13F primers.

**Estimation of viral load in hand rinse samples.** An RT-qPCR standard curve served as the basis for quantification of the viral load. The standard curve (corresponding to 49 to \( 4.9 \times 10^5 \) GEC) was constructed using serially diluted full-length NV genomic RNA that was transcribed from a full-length Norwalk virus cDNA clone. The concentration of the NV RNA transcript was determined as previously described (22). The approximate virus concentration in the hand rinse samples was calculated directly from the standard curve with corrections made to account for total sample volume.

**Statistical analysis.** NV concentrations in stool samples and hand rinse samples were expressed as \( \log_{10} \) GEC per gram of feces and total \( \log_{10} \) GEC per hand, respectively. The unpaired \( t \) test was applied to compare NV \( \log_{10} \) concentrations obtained from positive hand rinse samples with respect to volunteer infection status (infected versus uninfected) and sample collection time (i.e., during routine vital sign measurement, after defecation, or without clear sample collection information). Data were analyzed using SAS 9.2 (SAS, Cary, NC), and \( P \) values < 0.05 were considered statistically significant.

**RESULTS**

**Limits of detection for the EasyMAG/RT-qPCR assay.** The limit of detection of the RT-qPCR method used in the screening assay was 4.9 GEC per reaction. The limit of detection for the combined virus concentration-RNA extraction method was determined by seeding approximately \( 7.1 \times 10^1 \) to \( 7.1 \times 10^5 \) GEC of serially diluted (20%) NV-positive stool suspensions into 25 ml of glove juice solution and processing through RT-qPCR in triplicate trials. The lowest detectable concentration was considered the limit of detection for the combined assay and was equivalent to approximately \( 1.4 \times 10^2 \) (2.15 \( \log_{10} \)) GEC per 50 ml of hand rinse sample.

**Summary of NV screening results.** Based on the criteria of NV positivity and negativity, 125/159 (78.6%) of samples were negative and 34/159 (21.4%) were presumptively positive (data not shown) in the first trial. For all 34 presumptively positive samples and a subset of NV-negative samples (n = 36), the remaining rinse volumes were reprocessed by PEG precipitation and RNA extraction and then tested again by RT-qPCR amplification (with and without IAC) with duplicate amplifications. All negative samples remained negative. The final number of positive samples was reduced from 34 to 20 (Table 3).

**Confirmation of NV-positive samples.** All 20 NV-positive and 14 representative NV-negative hand rinse samples in the second round of testing were tested by conventional RT-PCR followed by dot blot hybridization with genogroup 1 (GI)-specific probes. All of the samples that were positive by RT-qPCR were also positive by conventional RT-PCR followed by hybridization. In general, there was a reverse correlation between the RT-qPCR \( C_T \) value and the intensity of the hybridization signal (Fig. 1). An additional 8 samples that were negative by RT-qPCR in round 2 of testing were weakly positive by RT-PCR and hybridization. Two hand rinse samples that tested positive for NV by RT-qPCR \( (C_T \) values were 26 and 28, respectively), along with the corresponding (same-day) stool samples, were reamplified by conventional RT-PCR, and the PCR products were cloned and sequenced. The NV sequences of the amplified PCR products in these hand rinse sam-

---

**Table 2.** RT-PCR primers and probes used for detection and confirmation of NV in hand rinse samples.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Primer sequence</th>
<th>Probe sequence</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnostic RT-qPCR</td>
<td>NVKS1-Forward: 5’-ACAGCATGGGACTCAACACA-3’</td>
<td>NVKS3 probe: 5’-(FAM)TCACCAGAATTGGCCGAGGTTGT(BHQ-1)-3’</td>
<td>Liu et al. (20)</td>
</tr>
<tr>
<td></td>
<td>NVKS2-Reverse: 5’-GGGAAGTACATGGGAATCCA-3’</td>
<td>IAC probe: 5’-(TET)ATCTCAGTTCGGTGTAGGTCGTTCGCTCC(BHQ-1)-3’</td>
<td>This study</td>
</tr>
<tr>
<td>Confirmation RT-PCR</td>
<td>COG1F: 5’-CGYTGGATGCGNTTYCATGA-3’</td>
<td>Ring a: 5’-(FAM)AGATYGCGATCYCCTGTCCA(TAMRA)-3’</td>
<td>Kageyama et al. (21)</td>
</tr>
<tr>
<td></td>
<td>COG1R: 5’-GTGAAGAGGCACCTCTAAGCA-3’</td>
<td>Ring b: 5’-(FAM)AGATCGCGGTCTCCTGTCCA(TAMRA)-3’</td>
<td>This study</td>
</tr>
</tbody>
</table>

*FAM, 6-carboxyfluorescein; TET, tetrachlorofluorescein; BHQ-1, black hole quencher 1; TAMRA, 6-carboxytetramethylrhodamine.*
ples were confirmed to be Norwalk virus sequences and were identical to those sequences isolated from the same subjects’ stool samples (data not shown).

Interpretation of NV test results. Based on RT-qPCR results, there was evidence of NV contamination in 25.4% (18/71) of hand rinse samples obtained from infected subjects, with an average titer of 3.86 (range of 3.30 to 4.45) log_{10} GEC per hand. Interestingly, subject 36 (the infected subject from whom the largest number of hand rinse samples were collected) had a high frequency (12/22 [54.5%]) of NV hand contamination, while subjects 34 and 46 had relatively lower positive rates (2/8 [25%]). Subject 36 was particularly interesting because of the high proportion of NV-positive hand samples (six samples) that were negative by RT-qPCR but positive by DNA hybridization. No evidence of NV contamination was identified on the hands of two infected subjects (subjects 37 and 40), although there was trace evidence of hand contamination in one of the rinses from subject 37 when the samples were screened by conventional RT-PCR followed by DNA hybridization (Table 3).

With respect to sample type, NV was detected in 37.5% (6/16) of samples collected during routine vital sign measurements, with an average of 3.32 log_{10} GEQ per hand. That proportion of NV-positive samples and that mean titer were significantly higher ($P < 0.05$) than the proportion and mean titer of the NV-positive samples (12.4% [11/89]; 2.30 log_{10}) collected immediately after bathroom use. In contrast, the proportion of NV-positive samples without clear collection information was 25% (10/40), with an average titer of 2.46 log_{10} GEC, and there were no statistically significant differences ($P > 0.05$) from the proportions and mean titers of positive samples collected during routine vital sign measurements and after bathroom use.

Surprisingly, NV was also identified in 2/88 (2.3%) hand rinse samples (and in one additional sample by RT-PCR and hybridization) obtained from uninfected subject 42 (Table 3). The mean log_{10} NV GEC of the positive samples derived from this uninfected subject was 2.81, which was significantly lower ($P < 0.05$) than the viral load estimated from the positive samples obtained from NV-infected subjects.

Relationship between clinical symptoms, viral shedding in stool, and hand contamination. Fig. 2 shows the temporal relationship between NV contamination on hands, occurrence of clinical symptoms, and virus shedding in stool. NV RNA was detected in 18 of 71 (25.4%) hand rinse samples collected from 4 of 6 (66.7%) subjects who were experiencing diarrhea and/or vomiting and who were also excreting NV in their stool on the day of hand sample collection. Surprisingly, NV was also identified in hand rinse samples from subject 42, who demonstrated diarrhea and/or fever during the time of hand rinse sample collection but for whom we were unable to confirm NV shedding or seroconversion despite repeated testing and multiple RT-PCR attempts.

### TABLE 3 Clinical symptoms, seroconversion, and RT-PCR detection of NV in stool samples and hand rinse samples collected from 12 subjects

<table>
<thead>
<tr>
<th>Subject status</th>
<th>Subject ID</th>
<th>Illness</th>
<th>Seroconversion</th>
<th>Stool sample result</th>
<th>Hand rinse sample result</th>
<th>Mean log_{10} (no. of GEC/hand)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
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<td>Yes</td>
<td>Pos</td>
<td>Pos</td>
<td>3.94</td>
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<td></td>
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<td>Yes</td>
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<td>Pos</td>
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<td>Yes</td>
<td>Pos</td>
<td>Pos</td>
<td>4.54</td>
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<td>Uninfected</td>
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<td>No</td>
<td>Neg</td>
<td>Neg</td>
<td>2.81</td>
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<td></td>
<td>41</td>
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<td>No</td>
<td>Neg</td>
<td>Neg</td>
<td>2.81</td>
</tr>
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<td></td>
<td>42</td>
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<td>No</td>
<td>Neg</td>
<td>Neg</td>
<td>2.81</td>
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<tr>
<td></td>
<td>44</td>
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<td>2.81</td>
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<td>No</td>
<td>Neg</td>
<td>Neg</td>
<td>2.81</td>
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<tr>
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<td>55</td>
<td>No</td>
<td>No</td>
<td>Neg</td>
<td>Neg</td>
<td>2.81</td>
</tr>
</tbody>
</table>

* a Illness, ≥3 loose or watery stool samples and/or any vomiting within 24 h.
* b ≥4-fold difference between the prechallenge and convalescent-phase serum NV-specific IgG titers.
* c Pos, positive; Neg, negative; Hyb, hybridizations.
* d At least one sample was confirmed positive by sequencing.
DISCUSSION

To our knowledge, this is the first direct experimental evidence of NoV contamination on the hands of individuals with known NV exposure in a controlled setting. A total of 25.4% of hand rinse samples obtained from 4 of 6 infected subjects showed evidence of NV contamination. The infected subjects were all secretor positive, experienced clinical symptoms, and exhibited NV shedding in fecal specimens and NV-specific IgG seroconversion. Low levels of NV contamination were found in hand rinses from 1 of 6 uninfected volunteers.

Not unexpectedly, interpretation of RT-qPCR results from the hand rinse samples was not always straightforward. Specifically, even for highly contaminated samples, $C_T$ values were rarely below 32. For this reason, presumptively positive samples were tested twice and subjected to multiple amplification reactions and confirmatory tests. Based on the criterion that both replicate samples must yield RT-qPCR values of $C_T \leq 38$, 18 of these samples were confirmed positive. As previously reported by others, it is possible that very high $C_T$ values in some samples actually corresponded to very low levels of NV contamination (23). The value of the confirmatory steps is illustrated by the fact that, for a select group of negative samples further tested by hybridization, some samples with average RT-qPCR $C_T$ values > 38 produced weak hybridization signals and a subset of 6 RT-qPCR negative samples were also weakly positive by hybridization.

NV contamination was also detected in two hand rinse samples from one uninfected subject (subject 42) who had no detectable NV shedding in his stool and no NV-specific IgG seroconversion but who did have the clinical symptoms of diarrhea and fever. These results were confirmed by replicate testing of hand rinse samples, two different RT-PCR protocols, and DNA hybridization. A previous study conducted in a diarrheal disease treatment center reported evidence of rotavirus contamination in the hand washings of attendants caring for patients without rotavirus, although other patients in the facility did indeed have rotavirus infection (24). While we do not know exactly how the hands of subject 42 became contaminated, contact with a contaminated fomite is a possibility. The potential for NoV surface contamination is well documented in outbreak settings (25, 26), and these viruses are readily transferred between surfaces and fingers (27). Hand rinse samples from subject 42 were collected in the same room 1 week after an episode occurred of virus shedding from a previous NV-infected subject in the same study. Although routine decontamination efforts had been performed in the ward environment between the two NV challenge events, NV environmental contamination can persist for long periods (6), and the viruses show resistance to many disinfectants (28).

This report has significance for both the health care and food service sectors. In health care, there is mounting evidence of the importance of hands (29) and surfaces (30) in the transmission of nosocomial infections. Likewise, the importance of hands in NoV transmission via food has been demonstrated through epidemiological investigation of outbreaks (10, 13), with more evidence provided recently (31). Efforts have also been undertaken to mathematically model the likelihood of NoV transmission in the food service environment (14). However, there are many unanswered questions that this study can help answer. For example, what degree of efficacy might be required for a hand sanitizer.
manufacturer to make specific antiviral claims? We do not yet have such standards in the United States, but in Canada and Europe, the standard is a 4 log₁₀ reduction (in vitro) and a 2 log₁₀ reduction (in vivo) (32) of the level of murine NoV, which may be more easily inactivated than human NoVs (33, 34). If, indeed, a dose of 1 to 2 log₁₀ NV GEC is enough to cause infection in susceptible individuals (22), and the hands of NV-infected individuals with symptoms harbor around 4 log₁₀ GEC, then a 2 log₁₀ reduction in vivo efficacy might not be sufficiently stringent to ensure adequate public health protection. A combination of hand washing and hand sanitizer may be more stringent, and a previous study reported that a regimen of hand washing with antibacterial soap followed by a 70% ethanol advanced-formula gel sanitizer achieved a 3.19 to 4.04 log₁₀ reduction of the level of cultivable surrogate murine NoV (35). If these findings could be translated into the removal of human NoV, the combined use of hand washing and hand sanitizer would provide added protection against transmission of human NoV.

The U.S. Food and Drug Administration (FDA) Food Code (36) for retail and food service settings recommends hand washing as a method to reduce transmission of food-borne pathogens from hands to food and other objects. The recommended hand-washing procedure includes washing hands using running warm water for at least 20 s, rubbing one hand against the other for 10 to 20 s, and finally rinsing and drying with towels or hot air. The FDA also suggests using gloves as a barrier to further prevent the transmission of pathogens. If these hand hygiene practices were properly used, opportunities for food contamination would be considerably reduced. However, factors such as improper use of gloves, loss of glove integrity, and, particularly, variable worker compliance can limit the effectiveness of Food Code hand hygiene interventions.

In conclusion, these laboratory results strongly support previous epidemiological evidence suggesting the importance of human hands in the transmission of NoV infection. The evidence of NV RNA on the hands of an uninfected individual suggests that man hands in the transmission of NoV infection. The evidence of NV RNA on the hands of an uninfected individual suggests that hands can become contaminated from NoV-contaminated surfaces. These results further support the need for more-effective hand hygiene agents and for continued emphasis on dedicated compliance with recommended hand hygiene practices.

ACKNOWLEDGMENTS

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We thank scientists David Macinga and Carrie Zapka from Gojo for their contributions to develop the hand rinse sample collection method and the nurses and Marshall Lyon at Emory Hospital for their help with hand rinse sample collection. We are grateful to Jean Leon, who led the clinical trial evaluating NV inactivation in shellfish by high pressure at Emory Hospital. We also thank Songli Xu, who helped with the statistical analysis in this study.

REFERENCES


