

Human Norovirus Transfer to Stainless Steel and Small Fruits during Handling

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ABSTRACT

Human noroviruses (NoVs) cause an estimated 58% of foodborne illnesses in the United States annually. The majority of these outbreaks are due to contamination by food handlers. The objective of this study was to quantify the transfer rate and degree of contamination that occurs on small fruits (blueberries, grapes, and raspberries) and food contact surfaces (stainless steel) when manipulated with NoV-contaminated hands. Human NoVs (genogroups I and II [GI and GII]) and murine norovirus (MNV-1) were inoculated individually or as a three-virus cocktail onto donor surfaces (gloved fingertips or stainless steel) and either immediately interfaced with one or more recipient surfaces (fruit, gloves, or stainless steel) or allowed to dry before contact. Viruses on recipient surfaces were quantified by real-time quantitative reverse transcriptase PCR. Transfer rates were 58 to 60% for GII NoV from fingertips to stainless steel, blueberries, and grapes and 4% for raspberries under wet conditions. Dry transfer occurred at a much lower rate (<1%) for all recipient surfaces. Transfer rates ranged from 20 to 70% from fingertips to stainless steel or fruits for the GI, GII, and MNV-1 virus cocktail under wet conditions and from 4 to 12% for all viruses under dry transfer conditions. Fomite transfer (from stainless steel to fingertip and then to fruit) was lower for all viruses, ranging from 1 to 50% for wet transfer and 2 to 11% for dry transfer. Viruses transferred at higher rates under wet conditions than under dry conditions. The inoculum matrix affected the rate of virus transfer, but the majority of experiments resulted in no difference in the transfer rates for the three viruses. While transfer rates were often low, the amount of virus transferred to recipient surfaces often exceeded 4- or 5-log genomic copy numbers, indicating a potential food safety hazard. Quantitative data such as these are needed to model scenarios of produce contamination by food handling and devise appropriate interventions to manage risk.

Human noroviruses (NoVs) are the leading cause of foodborne illnesses in the United States, contributing to an estimated 58% (~5.5 million) of illnesses annually (25). They are small (27 to 30 nm), nonenveloped, single-stranded RNA viruses belonging to the family *Caliciviridae*. Five genogroups (GI to GV) and more than 29 genotypes within these genogroups have been identified (30). Among this diverse group of viruses, the two primary genogroups that infect humans are GI and GII. GII genotype 4 (designated GII.4) NoVs are the most common genotypes found in humans. Variant strains of GII.4 NoVs emerge every 2 to 10 years and have been responsible for several pandemics (17, 26). GI viruses (such as GI.3b NoV) do not cause as many outbreaks but are commonly detected in foodborne outbreaks and in environmental waters (10, 29).

Illness caused by human NoV is marked by symptoms of acute gastroenteritis, including watery diarrhea, vomiting, nausea, abdominal cramps, and sometimes a low-grade fever. Individuals infected with human NoV may have all of these symptoms, some symptoms (i.e., vomiting only or diarrhea only), or no symptoms at all. Human NoV is generally nonlethal, although it can be lethal if severe dehydration

results from fluid loss. Virus can be shed in vomit and feces (from both ill and asymptomatic persons) and is normally transmitted through direct contact with ill individuals or indirect contact with food, water, or environmental surfaces that have been contaminated with virus. Viral shedding in both symptomatic and asymptomatic individuals has been reported at concentrations up to 10^{11} virus per gram of feces, which is remarkable considering the low 50% infectious dose, estimated to be ~18 virus particles (1, 27). In addition, as many as 3×10^7 virus particles may be present in a 30-ml bolus of vomit, which can aerosolize and disperse during vomiting (9). Facilitating indirect transmission, human NoV can survive on surfaces, on foods, and in water for long periods of time, ranging from days to even months, depending on the level of moisture and organic material in the surrounding environment (2, 8, 11, 13). They are moderately resistant to surface treatment with many common chemical disinfectants and to ethanol-based hand sanitizers (15, 18, 19). In the absence of a robust culture assay for human NoV, surrogate viruses that are similar to NoVs in terms of genetic structure and organization are used in infectivity studies, the most common of which are murine norovirus (MNV-1) and feline calicivirus (FCV) (8).

Small fruits are highly susceptible to contamination by human NoV as they are commonly harvested, packed, or

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prepared by hand. While mechanical harvesting is possible for fruits like blueberries and grapes, equipment accessibility may be lacking in smaller-sized operations. Delicate fruits like raspberries are often harvested with bare hands. Several outbreaks of human NoV have been associated with fresh or frozen raspberries suspected to have been contaminated at the farm level (3, 14, 20, 24). Human NoV is also the leading cause of produce-related outbreaks involving unspecified fruits (12). Produce can become contaminated directly by ill (or asymptomatic) food handlers practicing inadequate hand sanitation or indirectly after a healthy worker contacts a contaminated surface, such as a restroom door handle or water tap. Fomite contamination is strongly associated with human NoV outbreaks occurring in confined or institutional settings (16, 22). A recent study among catering companies also found that human NoV was present more frequently on environmental surfaces (kitchens and staff bathrooms) contacted by personnel from catering companies that reported an outbreak of human NoV than on surfaces contacted by personnel from catering companies with no reported outbreaks, suggesting a possible role of fomites in outbreaks of foodborne disease (7).

The transfer of NoV between fingertips and foods or food contact surfaces and between food contact surfaces and foods has been studied primarily using the surrogate virus FCV (6, 13). In one such study, the transfer of FCV from stainless steel surfaces to lettuce was compared with that of human NoV (GI.1), although lack of quantitative detection methods for human NoV and differences in the inoculation matrix used for the two viruses complicates making a direct comparison of the transfer rates of the two viruses (13). In another study, human NoV (GII) was used to demonstrate a high frequency of virus transfer from contaminated fomites to fingertips and then to household surfaces, although the results were not quantitative and transfer rates were not calculated (4).

In this study, quantitative transfer data for human NoV and the widely-used surrogate MNV-1 were generated and compared. The amount of virus transferred and the percentage of virus transferred between gloved fingertips and food contact surfaces (stainless steel) or fruits (blueberries, grapes, and raspberries) was determined for GII.4 NoV alone or using an inoculum cocktail containing both human NoV (GII.4 and GI.3b) and MNV-1. Experiments were performed under wet transfer conditions and after allowing the virus inoculum to dry before transfer. Secondary transfer from fingertips to a stainless steel (intermediate) surface and then to fruits and indirect contamination of fruits after touching a contaminated fomite (stainless steel) were also investigated. Using the three-virus cocktail, the impact of the inoculum matrix on virus transfer was addressed and the acceptability of MNV-1 as a surrogate for transfer studies was examined. Quantitative data such as those generated in this study are needed to model scenarios of produce contamination by food handling and devise appropriate interventions to manage risk.

MATERIALS AND METHODS

Virus stock preparation. Stool specimens from an outbreak of human NoV GII.4 (a gift of Andrea Maloney, South Carolina

Department of Health and Environmental Control, Columbia) and an outbreak of GI.3b NoV (provided by Dr. Jan Vinjé at the Centers for Disease Control and Prevention, Atlanta, GA) were obtained. Each was prepared in a 20% (wt/vol) suspension in phosphate-buffered saline (PBS), pH 7.2, vortexed for 30 s, and clarified by centrifugation at $14,000 \times g$ for 10 min in a benchtop microcentrifuge. MNV-1 (a gift from Dr. Herbert W. Virgin, Washington University School of Medicine, St. Louis, MO) was cultured in RAW 264.7 cells (ATCC TIB-71) in Dulbecco's modified Eagle's medium supplemented with 10% low-endotoxin fetal bovine serum (FBS) (HyClone, Logan, UT) as described previously (8). For MNV-1, a three-times freeze-thawed cell culture lysate was prepared by centrifugation at $2,000 \times g$ for 15 min at 4°C, filtration of the supernatant using a 0.2- μ m-pore-size filter (Millipore, Inc., Bedford, MA), and concentration by ultracentrifugation at $100,000 \times g$ for 1 h at 4°C before overnight resuspension of the pellet in PBS. The 20% stool suspension of GII.4 NoV was used as the "high-titer GII.4" inoculum and contained $\sim 10^{11}$ genome copies per ml. A lower titer three-virus cocktail was prepared using the GI.3b 20% stool suspension plus a dilution of the high-titer GII.4 inoculum and a dilution of the ultracentrifuged MNV-1 stock. This lower titer cocktail contained $\sim 10^8$ genome copies of each virus type per milliliter. In experiments where the inoculum matrix was an experimental variable, a different set of virus stocks was prepared. Two stool specimens testing positive for GI NoV (and negative for GII NoV) or testing positive for GII NoV (but negative for GI NoV) by real-time reverse transcriptase PCR (RT-PCR) were prepared in 20% fecal suspensions. Fecal suspensions were prepared by centrifugation at $9,000 \times g$ for 30 min at 4°C, filtration of the supernatant using a 0.45- μ m-pore-size filter and a 0.2- μ m-pore-size filter (Millipore), and concentration by ultracentrifugation at $100,000 \times g$ for 1 h at 4°C before overnight resuspension of the pellet in PBS containing 5% FBS. Ultracentrifuged MNV-1 stocks were prepared as indicated above, but the pellet was suspended in PBS containing 5% FBS. For ultracentrifuged virus stocks, a cocktail of ultracentrifuged human NoVs and MNV-1 were combined. For negative stool virus stocks, a (human) fecal specimen (10%, wt/vol) testing negative for both GI and GII NoV was added to these ultracentrifuged virus stocks. A 20% stool suspension was prepared and clarified as described above. For the untreated virus stock, 20% clarified stool suspensions and ultracentrifuged MNV-1 were combined. These virus stocks were adjusted to $\sim 10^8$ genome copies of each virus per ml. Aliquots (50 to 100 μ l) of all virus stocks were prepared prior to storage at -70°C .

RNA extraction and quantification. Viral RNA was extracted using a viral RNA minikit (Qiagen, Valencia, CA) and amplified in individual reaction mixtures by real-time RT-PCR (StepOne, Applied Biosystems, Foster City, CA) using NoV-specific primers and probes (23, 28) and a QuantiTect probe one-step RT-PCR kit (Qiagen). Viral RNA was quantified by comparison to a standard curve of NoV RNA transcripts of a known concentration. RNA transcripts (gifts from Dr. Jan Vinjé at the Centers for Disease Control and Prevention) were prepared by *in vitro* transcription (MEGAscript T7 kit, Invitrogen, Life Technologies, Grand Island, NY) of a 3-kb clone of NoV genomes (GI.1 and GII.4) that included the open reading frame 1–open reading frame 2 junction or a full-length clone of the MNV-1 genome. Degradation of residual DNA by triple digestion with TURBO DNase (included in the MEGAscript kit) was confirmed by PCR amplification. GII.4 RNA transcripts were used as internal amplification controls, spiked into RT-PCR reaction mixtures containing extracts (Qiagen viral RNA minikit) obtained from negative control samples. Negative controls were processed identically to the recovery controls but were not inoculated with NoVs.

Preparation of food items and food contact surfaces. All fruits (raspberries, red grapes, and blueberries) were soaked in a 10% bleach solution for 10 min, rinsed three times in Milli-Q sterile deionized water, dried for 30 min, and exposed to germicidal UV for 10 min in a sterile weigh boat before use. Stainless steel coupons (5 cm by 2 cm, finish no. 4) were soaked in 70% ethanol for 1 h, rinsed with Milli-Q sterile water, and then autoclaved at 121°C for 30 min at 17 lb/in² and stored in a sterile beaker until use. Latex gloves (Diamond Grip Plus, powder-free latex exam gloves, Microflex, Reno, NV) were exposed to germicidal UV for 10 min on each side prior to use.

Virus inoculation and transfer. The starting item (donor) for each transfer was inoculated with 10 1- μ l iterations of stool suspension and either allowed to dry for 30 min, representative of a dry inoculation procedure, or used immediately during studies with a wet inoculation procedure. The index fingers of latex gloves were cut out and placed on the bottom portion of a 15-ml centrifuge tube during inoculation. The gloved 15-ml centrifuge tube served as a gloved fingertip during transfer studies. In studies using the cocktail of GI, GII, and MNV-1 as the inoculum, the three viruses were combined and coinoculated onto each donor surface. Pressure was applied during each transfer experiment with 50 \pm 5 g of pressure for 5 s. Pressure was monitored by placing each recipient item on an electronic scale and taring the scale before the pressure was exerted by the donor surface. The area of contact between donor and recipient surfaces was approximately a 1-cm² area.

Three scenarios of food handler contamination of produce were mirrored in the experiments in this study: (i) single transfer, involving stainless steel and fruits as recipient surfaces after direct transfer from gloved fingertips, (ii) “with intermediate” transfer, involving inoculated fingertips touching first a stainless steel intermediate and then fruits using the same fingertip, and (iii) fomite transfer, occurring when previously clean gloves touch an inoculated stainless steel surface, becoming contaminated in the process, and then go on to contact fruits.

Virus elution from recipient surfaces. After viral transfer, stainless steel coupons and gloved fingertips were placed in a 50-ml tube containing 10 ml of 0.1 M PBS with 1 M NaCl, pH 7.2, and vortexed for 30 s. A 200- μ l aliquot was transferred to a sterile Eppendorf tube for RNA extraction. Fruit was placed in a sterile 50-ml centrifuge tube containing 10 ml of 0.1 M PBS with 0.05% (vol/vol) Tween 20 and 1 M NaCl, pH 7.2. For blueberries and grapes, virus was eluted by vortexing the centrifuge tube for 30 s. Virus was eluted from raspberries by placing the fruit in a 50-ml centrifuge tube and inverting the tube upside down 30 times. A 200- μ l aliquot of virus eluate from all fruits underwent RNA extraction. All samples of extracted RNA were either used immediately for RT-PCR or stored at –80°C before testing. Due to the dilution factor, the lower limit of detection for the recovery procedure was 2,500 genomic copy numbers (or 3.4 log of virus) for all experiments involving virus transfer.

Calculations and statistical analysis. All experiments involving transfer between only two objects (donor and recipient) were performed with three replicates with duplicate samples in each replicate. For experiments involving two transfer steps (donor to an intermediate surface and then to recipient), six repetitions were made with duplicate samples in each replicate. Recipient surfaces inoculated directly with 10 μ l of virus stock served as recovery controls and were used to determine the average recovery percentage for each recipient surface type using the following equation:

TABLE 1. Average percentages of recovery of GI.3b and GII.4 NoV or MNV-1 from items used in transfer experiments

Inoculum	Pathogen recovered	Recipient	% recovery \pm SD	
			Wet	Dry
GII	GII.4	Stainless steel	93 \pm 11	90 \pm 3
Cocktail	GI.3b	Stainless steel	104 \pm 24	44 \pm 12
Cocktail	GII.4	Stainless steel	111 \pm 33	82 \pm 10
Cocktail	MNV-1	Stainless steel	99 \pm 11	81 \pm 22
GII	GII.4	Gloved finger	87 \pm 7	51 \pm 19
Cocktail	GI.3b	Gloved finger	83 \pm 14	42 \pm 19
Cocktail	GII.4	Gloved finger	89 \pm 17	33 \pm 8
Cocktail	MNV-1	Gloved finger	104 \pm 22	50 \pm 22
GII	GII.4	Blueberry	72 \pm 14	53 \pm 21
Cocktail	GI.3b	Blueberry	57 \pm 6	34 \pm 22
Cocktail	GII.4	Blueberry	101 \pm 15	44 \pm 11
Cocktail	MNV-1	Blueberry	74 \pm 17	60 \pm 18
GII	GII.4	Grape	108 \pm 14	65 \pm 15
Cocktail	GI.3b	Grape	77 \pm 11	32 \pm 15
Cocktail	GII.4	Grape	95 \pm 29	14 \pm 12
Cocktail	MNV-1	Grape	60 \pm 8	31 \pm 7
GII	GII.4	Raspberry	25 \pm 8	15 \pm 4
Cocktail	GI.3b	Raspberry	64 \pm 32	15 \pm 8
Cocktail	GII.4	Raspberry	42 \pm 10	9 \pm 4
Cocktail	MNV-1	Raspberry	54 \pm 11	5 \pm 3

$$\% \text{ recovery} = \frac{\text{virus titer recovered}}{\text{virus titer inoculated}} \times 100\%$$

For transfer experiments, the genomic copy numbers of each virus recovered from recipient surfaces were adjusted by the recovery percentage using the following equation:

$$\% \text{ transfer} = \frac{\text{virus titer recovered from recipient}}{\% \text{ recovery}} \times 100\%$$

For statistical analysis, the responses (percentage transferred or the logarithmic value of virus titer recovered from recipient surface) among the various levels of each treatment were determined by analysis of variance using SAS software (version 9.2, SAS Institute Inc., Cary, NC). Significance was determined at the $\alpha = 0.05$ level. Values obtained from different combinations of factors were analyzed by using a generalized linear model. Significant differences in least-square means are indicated if the *P* value was <0.05.

RESULTS

Percentages of NoV recovered from recipient surfaces. To determine the effectiveness of the elution protocols, the percentages of recovery of virus from stainless steel coupons, gloved fingertips, and fruits were determined (Table 1). Each of these surfaces served as a recipient surface during the transfer experiments described below. Recovery percentages were determined for each item using either the high-titer GII.4 NoV inoculum ($\sim 10^9$ genomic copies per 10- μ l inoculum) or the GI.3b, GII.4, and MNV-1 cocktail inoculum that was lower in titer ($\sim 10^6$ genomic copies of each virus per 10- μ l inoculum). Each item was either processed immediately after inoculation (wet) or allowed to dry for 30 to 40 min (dry) before processing. The average recovery percentages for at least three replicate trials per inoculum-surface pair ranged from 93 to 111% for stainless

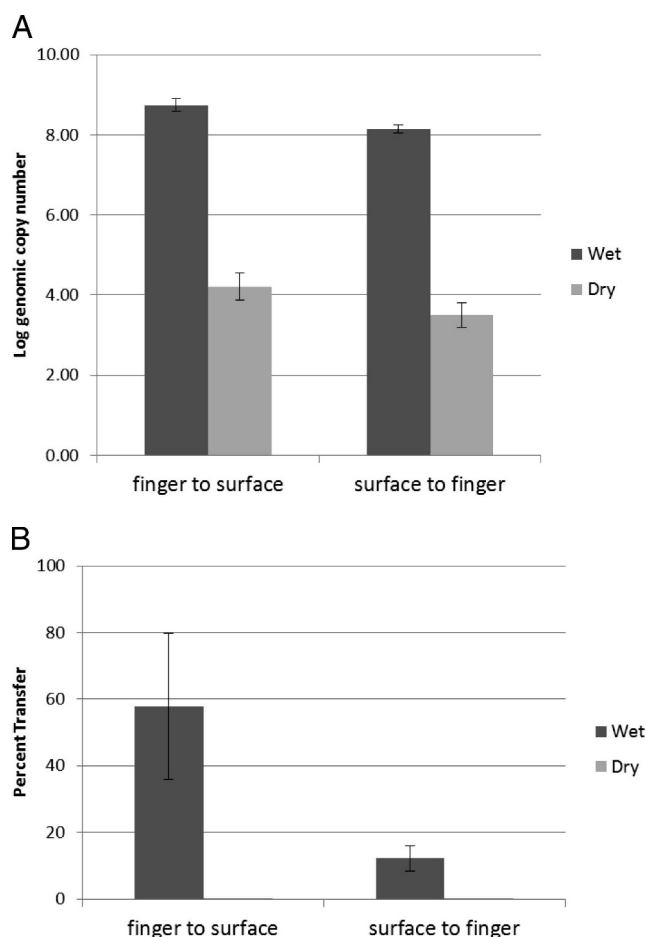


FIGURE 1. Transfer of high-titer GII.4 NoV between latex-gloved fingertips and stainless steel surfaces under wet and dry conditions of transfer. (A) Log genomic copy numbers detected on recipient surfaces. (B) Percentages transferred between donor and recipient surfaces.

steel coupons and 83 to 104% for the fingertips of latex gloves when the inoculum was wet and 44 to 90% and 33 to 51% for stainless steel and latex gloves, respectively, when the inoculum was allowed to dry. For blueberries, the average recovery percentages were 57 to 104% and 34 to 60% for wet and dry inoculation procedures. Similar recovery percentages of 60 to 108% and 14 to 65% were obtained for wet and dry inoculation procedures on grapes. Recovery of virus from raspberries was more difficult, especially when the inoculum was allowed to dry. The average recovery percentages ranged from 25 to 64% when wet and 5 to 15% for the dried inoculum on raspberries. There was no difference in the recovery percentages that was attributable to the titer of the inoculum or the type (genogroup) of virus used. However, recovery percentages were greater for the wet inoculation procedure.

High-titer GII.4 NoV transfer between gloved fingertips and stainless steel. Contamination of stainless steel surfaces by contact with NoV-contaminated gloves or contamination of gloves by contact with NoV on stainless steel was simulated in transfer experiments. Donor surfaces were inoculated with high-titer GII.4 NoV and either immediately interfaced with or allowed to dry for 30 to

40 min before interfacing with a recipient surface for 5 s using 50 g of pressure. The amounts of virus (log genomic copy numbers) detected on recipient surfaces after transfer of GII.4 NoV from donor surfaces are depicted in Figure 1A. Averages (\pm standard deviations) of 8.74 (\pm 0.16) and 4.21 (\pm 0.33) log genomic copy numbers were detected on stainless steel surfaces after transfer from inoculated latex glove fingertips following wet and dry inoculation procedures (Fig. 1A). The average percentages transferred were 58% (\pm 22%) for wet transfer and <0.01% for dry transfer (Fig. 1B). Virus detected on gloved fingertips after transfer from stainless steel averaged 8.14 (\pm 0.11) and 3.50 (\pm 0.31) log genomic copy numbers under wet and dry transfer conditions, respectively (Fig. 1A). The percentages transferred ranged from 14% (\pm 4%) for wet transfer to <0.01% for dry transfer experiments (Fig. 1B). In all cases, dry transfer of GII.4 NoV occurred to a lesser degree than wet transfer ($P < 0.0001$). Transfer experiments for which gloved fingertips were the recipients yielded significantly smaller amounts of GII.4 NoV under both wet ($P = 0.018$) and dry ($P < 0.001$) conditions than did experiments with stainless steel recipients.

High-titer GII.4 NoV transfer from gloved fingertips to small fruits. GII.4 NoV transfer from artificially contaminated gloved fingertips to blueberries, grapes, and raspberries was determined in a similar set of experiments, where fruits were contacted for 5 s with 50 g of pressure using wet or dry inoculation procedures. The average log genomic copy numbers of virus detected on recipient blueberries were 8.69 (\pm 0.35) under wet and 4.89 (\pm 1.71) under dry conditions of inoculation (Fig. 2A). Grapes harbored averages of 8.75 (\pm 0.13) and 2.75 (\pm 0.46) log genomic copy numbers after wet and dry transfer experiments, and 7.61 (\pm 0.12) and 3.19 (\pm 1.16) log genomic copy numbers of GII.4 NoV were detected on recipient raspberries after wet and dry transfer (Fig. 2A). The percentages transferred under wet conditions were 60% (\pm 43%), 58% (\pm 17%), and 4% (\pm 1%) for recipient blueberries, grapes, and raspberries, respectively (Fig. 2B). Transfer under dry conditions was <1% for all recipient fruits (Fig. 2B), which was significantly lower than wet transfer for all fruits ($P < 0.001$). There were no differences in the percentages transferred to blueberries and grapes ($P = 0.924$) under wet conditions or between any of the fruits under dry transfer conditions (P values were greater than 0.989), but transfer to raspberries was significantly less under wet conditions than for blueberries ($P = 0.004$) or grapes ($P = 0.005$).

Transfer of a NoV cocktail between gloved fingertips and stainless steel. A NoV cocktail consisting of a mixture of human NoVs GI.3b and GII.4 and MNV-1 and containing $\sim 10^6$ genome copies of each virus per 10- μ l inoculum was created, and transfer experiments were repeated with this lower titer mixture of representative NoVs. The amounts of virus (log genomic copy numbers) detected on recipient surfaces after transfer of the NoV cocktail from donor surfaces are depicted in Figure 3A.

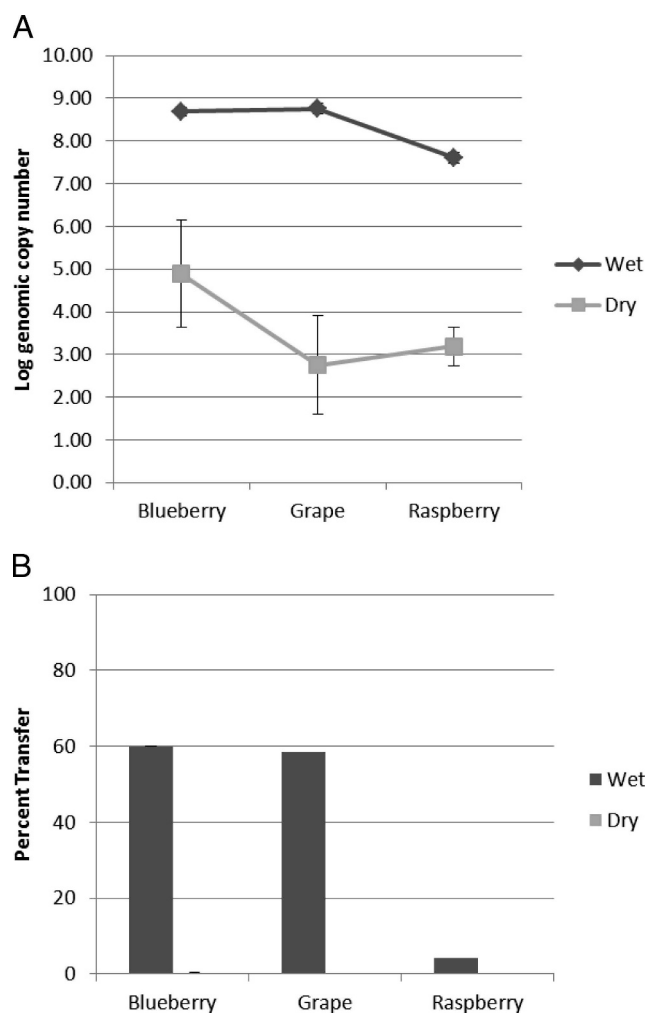


FIGURE 2. Transfer of high-titer GII.4 NoV between latex-gloved fingertips and small fruits under wet and dry conditions of transfer. (A) Log genomic copy numbers detected on recipient surfaces. (B) Percentages transferred between donor and recipient surfaces.

Averages of $5.41 (\pm 0.16)$, $5.71 (\pm 0.14)$, and $5.56 (\pm 0.36)$ log genomic copy numbers were detected on stainless steel surfaces after transfer from inoculated latex glove fingertips under wet inoculation procedures, and averages of $5.07 (\pm 0.21)$, $5.10 (\pm 0.34)$, and $4.30 (\pm 0.26)$ were detected on these surfaces after fingertip transfer under dry inoculation procedures for GI.3b, GII.4, and MNV-1, respectively (Fig. 3A). The average percentages transferred were 26% ($\pm 9\%$), 53% ($\pm 16\%$), and 44% ($\pm 27\%$) for wet transfer of GI.3b, GII.4, and MNV-1, respectively, and 12% ($\pm 6\%$), 5% ($\pm 4\%$), and 4% ($\pm 2\%$) for dry transfer of these viruses to recipient stainless steel coupons (Fig. 3B). Virus detected on gloved fingertips after transfer from stainless steel averaged $5.39 (\pm 0.13)$, $5.29 (\pm 0.15)$, and $5.31 (\pm 0.13)$ log genomic copy numbers under wet transfer conditions and $5.39 (\pm 0.20)$, $4.78 (\pm 0.20)$, and $5.22 (\pm 0.22)$ under dry transfer conditions for GI.3b, GII.4, and MNV-1, respectively (Fig. 3A). The percentages transferred ranged from 25% ($\pm 8\%$), 20% ($\pm 6\%$), and 21% ($\pm 7\%$) for wet transfer of GI.3b, GII.4, and MNV-1 to 26% ($\pm 10\%$), 6% ($\pm 3\%$), and 18% ($\pm 10\%$) for dry transfer of these viruses from gloved latex fingertips (Fig. 3B).

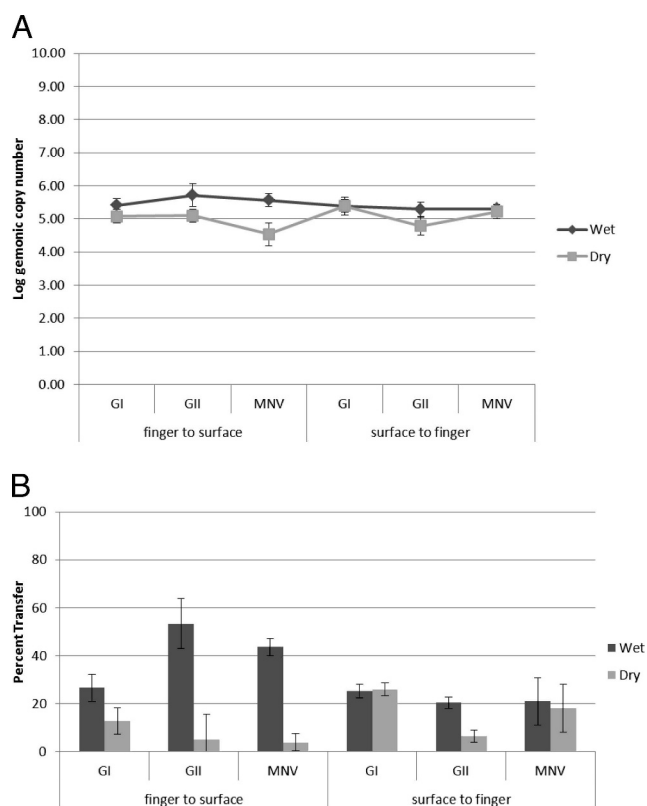


FIGURE 3. Transfer of NoV cocktail between latex-gloved fingertips and stainless steel surfaces under wet and dry conditions of transfer. (A) Log genomic copy numbers detected on recipient surfaces. (B) Percentages transferred between donor and recipient surfaces.

Overall, the transfer condition (dry or wet) was the only variable that had a significant impact on the number of viruses detected on recipient surfaces ($P < 0.001$) or the percentage of viruses transferred to recipient surfaces ($P < 0.001$). An interaction effect was also observed between transfer condition and donor type (stainless steel or latex glove fingertip) when the transfer percentage was considered ($P = 0.001$), but the virus type (GI.3b, GII.4, or MNV-1) was not a factor that significantly affected virus transfer ($P = 0.968$).

NoV cocktail detection on small fruits and transfer percentage following contact with gloved fingertips.

Experiments on NoV transfer from gloved latex fingertips to small fruits (blueberries, grapes, and raspberries) were repeated with the NoV cocktail ($\sim 10^6$ genome copies of GI.3b, GII.4, and MNV-1 per $10 \mu\text{l}$) as the inoculum. Additional transfer experiments were also conducted to examine (i) NoV transfer from gloved fingertips to fruits after first touching a stainless steel (intermediate) surface or (ii) fomite transfer from contaminated stainless steel surfaces to clean gloved fingertips and then to fruits touched by these newly contaminated gloves. The latter set of experiments was conducted to investigate fingertip contamination of fruits after contact with fomite surfaces. All transfer experiments were conducted using both a wet and a dry inoculum and under the same contact conditions as previous experiments.

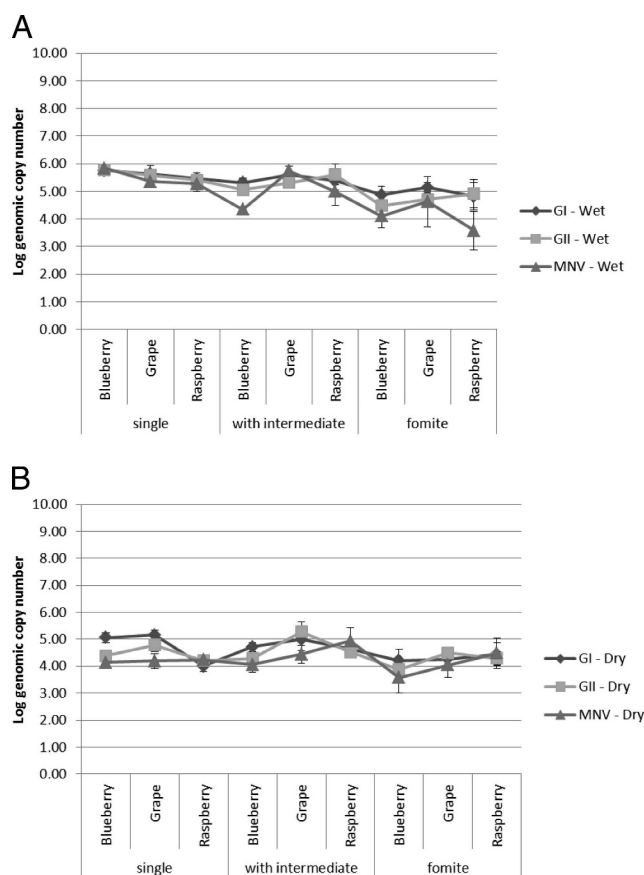


FIGURE 4. Transfer of NoV cocktail between latex-gloved fingertips and small fruits expressed as log genomic copy numbers detected on recipient fruit under wet (A) and dry (B) conditions of transfer.

After single-transfer experiments (from gloved fingertips to fruits), the average log genomic copy numbers of virus detected on recipient blueberries were 5.75 (± 0.16), 5.78 (± 0.03), and 5.84 (± 0.10) for GI.3b, GII.4, and MNV-1 under wet conditions (Fig. 4A). Grapes harbored averages of 5.63 (± 0.21), 5.58 (± 0.17), and 5.35 (± 0.27) log genomic copy numbers of GI.3b, GII.4, and MNV-1 after wet transfer experiments, and 5.45 (± 0.31), 5.41 (± 0.08), and 5.06 (± 0.22) log genomic copy numbers of GI.3b, GII.4, and MNV-1 were detected on recipient raspberries after wet transfer (Fig. 4A). After single-transfer experiments under dry conditions, the average log genomic copy numbers of virus detected on recipient blueberries were 5.06 (± 0.16), 4.39 (± 0.25), and 4.14 (± 0.28) for GI.3b, GII.4, and MNV-1 (Fig. 4B). Grapes harbored averages of 5.16 (± 0.20), 4.78 (± 0.22), and 4.19 (± 0.21) log genomic copy numbers of GI.3b, GII.4, and MNV-1 after dry transfer experiments, and 4.01 (± 0.19), 4.21 (± 0.24), and 4.24 (± 0.27) log genomic copy numbers of GI.3b, GII.4, and MNV-1 were detected on recipient raspberries after dry transfer (Fig. 4B). The percentages transferred under wet and dry conditions were also determined for each virus, as indicated in Figure 5A through 5C. The average percentages for wet transfer to fruits ranged from 33 to 59%, 26 to 60%, and 20 to 70% for GI.3b, GII.4, and MNV-1, while the average percentages for dry transfer to fruits ranged from 4 to 5% for all three viruses.

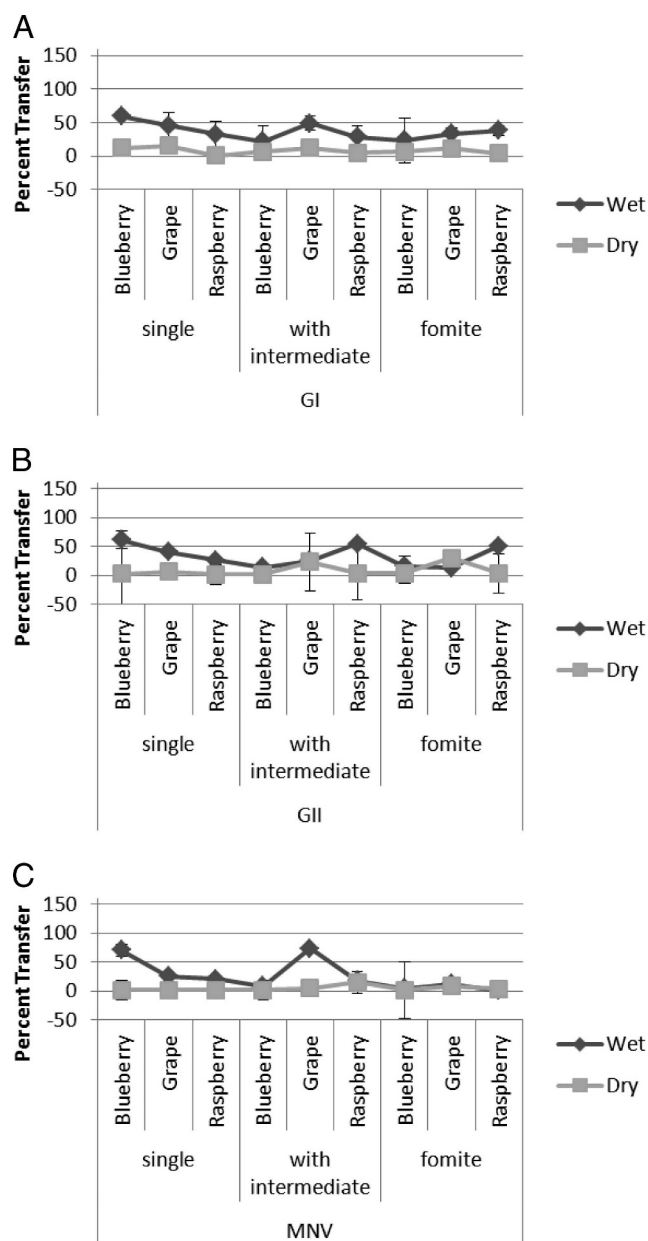


FIGURE 5. Transfer of NoV cocktail between latex-gloved fingertips and small fruits expressed as percentages of GI.3b (A), GII.4 (B), and MNV-1 (C) transferred to recipient fruit.

When gloved fingertips were inoculated with the NoV cocktail and interfaced first with a stainless steel (intermediate) surface before touching fruits, the average genomic copy numbers of GI.3b, GII.4, and MNV-1 viruses detected after wet transfer were 5.31 (± 0.18), 5.06 (± 0.28), and 4.34 (± 0.91) for blueberries, 5.59 (± 0.33), 5.31 (± 0.32), and 5.74 (± 0.41) for grapes, and 5.38 (± 0.32), 5.61 (± 0.37), and 4.99 (± 0.51) for raspberries, respectively. Under dry transfer conditions, the GI.3b, GII.4, and MNV-1 average log genomic copy numbers detected were 4.72 (± 0.32), 4.29 (± 0.17), and 4.06 (± 0.46) for blueberries, 4.99 (± 0.41), 5.29 (± 0.29), and 4.44 (± 0.57) for grapes, and 4.64 (± 0.27), 4.53 (± 0.20), and 4.94 (± 0.48) for raspberries, respectively (Fig. 4B). The percentages transferred under wet and dry conditions were also determined for each virus, as indicated in Figure 5A through 5C. The

average percentages for wet transfer to fruits after first touching a stainless steel intermediate ranged from 22 to 49%, 14 to 55%, and 8 to 73% for GI.3b, GII.4, and MNV-1, respectively, while the average percentages for dry transfer to fruits ranged from 6 to 13%, 2 to 23%, and 2 to 15% for GI.3b, GII.4, and MNV-1, respectively.

Finally, when stainless steel coupons were inoculated with the NoV cocktail and first interfaced with a clean latex-gloved fingertip before touching fruits, the average genomic copy numbers of respective GI.3b, GII.4, and MNV-1 detected after wet transfer were 4.86 (± 0.88), 4.48 (± 1.13), and 4.10 (± 0.96) for blueberries, 5.13 (± 0.76), 4.71 (± 0.74), and 4.63 (± 0.73) for grapes, and 4.82 (± 1.13), 4.91 (± 1.18), and 3.58 (± 0.57) for raspberries (Fig. 4A). Under dry transfer conditions, the average log genomic copy numbers detected were 4.21 (± 0.95), 3.88 (± 1.01), and 3.58 (± 1.06) for blueberries, 4.26 (± 1.21), 4.50 (± 1.36), and 4.04 (± 1.24) for grapes, and 4.45 (± 0.44), 4.29 (± 0.57), and 4.48 (± 0.41) for raspberries for GI.3b, GII.4, and MNV-1, respectively (Fig. 4B). The percentages transferred under wet and dry conditions were also determined for each virus, as indicated in Figure 5A through 5C. The average percentages for wet transfer to fruits by fomite contact (first touching a contaminated stainless steel surface with a previously uncontaminated glove) ranged from 24 to 38%, 16 to 50%, and 1 to 12% for GI.3b, GII.4, and MNV-1, while the average percentages for dry transfer to fruits ranged from 4 to 11%, 3 to 30%, and 2 to 8% for GI.3b, GII.4, and MNV-1, respectively.

Overall, under wet transfer conditions, there were no significant differences in the transfer percentages between recipient fruits ($P = 0.679$) or between viruses ($P = 0.188$), but there was a difference depending on the type of transfer (single, with intermediate, or fomite) ($P = 0.006$). The interaction between fruit and type of transfer was the only noted interaction of significance ($P < 0.001$). Under dry transfer conditions, there was a significant difference in transfer percentage between recipient fruits ($P = 0.001$), but there were no differences in transfer percentage between virus types ($P = 0.222$) or types of transfer ($P = 0.430$). The only interaction that was significant was that between virus and type of fruit ($P = 0.022$). When viruses were examined individually, the transfer percentage under dry conditions was less than the transfer percentage under wet conditions ($P < 0.001$) for all viruses. Fomite transfer occurred to a lesser degree than single transfer for all viruses, but virus transfer to fruits after first touching an intermediate surface was either similar to single transfer or similar to both single and fomite transfers (Fig. 5A through 5C).

When transfer scenarios were considered individually, the following trends were observed: (i) the virus transfer percentages obtained on fruit were highest for blueberries, then grapes, and then raspberries for single-transfer experiments (at a level of significance of $\alpha = 0.05$), but there were no differences in transfer percentages between the GI.3b, GII.4, and MNV-1 viruses ($P = 0.128$); (ii) when a stainless steel intermediate was included, the transfer

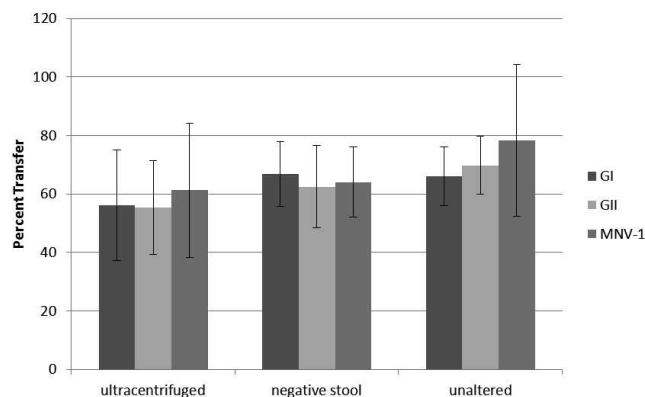


FIGURE 6. Impact of virus suspension matrix on percentages of NoV cocktail transferred between latex-gloved fingertips and stainless steel surfaces.

percentage was greatest for grapes, then raspberries, and then blueberries (at a level of significance of $\alpha = 0.05$), but again, no differences were observed in the transfer rates of the three viruses ($P = 0.992$); (iii) for fomite transfer experiments, there was no difference in transfer percentage due to the type of fruit ($P = 0.302$) or to the virus under dry transfer conditions, but under wet conditions, the transfer of MNV-1 was less than the transfer of GI.3b and GII.4 NoV (at a level of significance of $\alpha = 0.05$). Data trends can be observed in Figure 5A through 5C.

Impact of inoculum matrix on NoV transfer from gloved fingertips to stainless steel. To investigate whether the suspending medium (inoculum matrix) of the NoV cocktail affected the transfer of viruses between donor latex gloves and recipient stainless steel coupons, three different NoV cocktails were prepared. The suspending medium for the NoV cocktails consisted of either 5% FBS (ultracentrifuged), a 10% stool suspension from a fecal specimen testing negative for GI and GII NoV (negative stool), or a mixture of 10% stool preparations of GI and GII from clinical specimens and MNV-1 cell culture lysate. The average transfer percentages of GI, GII, and MNV-1, respectively, were 56% ($\pm 20\%$), 55% ($\pm 16\%$), and 61% ($\pm 23\%$) for the ultracentrifuged cocktail, 66% ($\pm 9\%$), 63% ($\pm 14\%$), and 64% ($\pm 12\%$) when a negative stool suspension was included in the inoculum, and 66% ($\pm 10\%$), 70% ($\pm 10\%$), and 78% ($\pm 26\%$) with an unaltered cocktail as the inoculum (Fig. 6). There were no significant differences in the transfer percentages of the three viruses regardless of the inoculum matrix ($P = 0.442$). However, the inoculum matrix did affect the rate of virus transfer, as indicated in Figure 6. The unaltered virus cocktail prepared in this set of experiments was most similar to the three-virus cocktail used in the studies described above. When comparing the new unaltered cocktail inoculum to the cocktail used in the experiments described above, the viruses in the new preparation transferred to recipient stainless steel surfaces to a higher degree (measured by log genomic copy numbers recovered from recipient surfaces and by transfer percentage) than did viruses in the other study ($P < 0.001$).

DISCUSSION

Food workers can contaminate food either through fomites or direct contact. Hands or gloves can become contaminated with pathogens after contact with previously contaminated fomite surfaces and food, following restroom use, or after vomiting. Improper hand sanitation in restroom facilities not only allows for some pathogens to remain on the hands of individuals in high quantities but also presents the risk for leaving pathogens on contact surfaces, such as door handles, which can transfer to healthy individuals upon subsequent contact. Few studies address the likelihood and degree of food contamination that occurs with human NoV during food handling. For the few studies conducted with the FCV surrogate, direct comparisons between the transfer rates of human NoV and the surrogate were either not made or were not possible due to differences in the inoculum matrix or the (semiquantitative) methods used for virus detection (6, 13). The purpose of this research was to quantify the degree of contamination by human NoV that occurs during handling of fruits, including blueberries, grapes, and raspberries, under wet and dry transfer conditions and with different initial contamination loads. Such information is important for constructing models for the assessment and management of risk. In addition, the human NoV surrogate, MNV-1, was included in the inoculum cocktail with the human NoV GI and GII strains to determine its acceptability for use in future transfer studies and to address the impact of the suspension matrix on virus transfer.

Transfer of virus was higher under wet than under dry conditions for nearly all scenarios and in nearly all comparisons between NoV genogroups. Since it has been observed in previous literature that transfer of a pathogen to food is higher when the inoculum has not been allowed to dry (13, 21), it must be noted that wet transfer may not be uncommon in the practical setting. Scenarios where wet transfer may occur include food worker contamination of door handles or other bathroom surfaces before exiting the restroom. After a restroom visit, a food worker, not respecting hygienic practices, may immediately or within a short period of time (<30 min) begin to handle foods, not allowing sufficient time for contaminated hands to dry. Alternatively, a food worker may use the restroom immediately following an ill person. Contact with bathroom surfaces may result in the transfer of viruses to previously unsoiled hands. Indirect transfer to foods then becomes possible. Study results indicate that drying hands and avoiding contact with wet surfaces in the restroom significantly decrease, although they do not completely eliminate, the risk of contaminating foods after restroom use. Hand washing and bathroom sanitation should be strictly enforced to further minimize this risk.

Studies revealed that the potential for contamination of fresh produce is high if virus transfer from contaminated fingertips to fruit is uninterrupted. In scenarios where an intermediate stainless steel surface was included, the transfer percentages were similar to the results of single-transfer experiments. However, indirect transfer from

fingertips to fruits after contamination by contact with a stainless steel fomite was significantly lower than direct transfer. It should be noted, however, that when the starting contamination level is very high, even low percentages of transfer can result in moderate levels of contamination. With a 50% infectious dose of 18 NoV particles needed to cause illness (27), even low levels of contamination may still present a health risk.

There was a high degree of variability of results that increased with experiments involving more than one transfer event before virus detection on fruit. Logarithmic conversion of the genomic copy number data was necessary for statistical analysis. The recovery percentages for each recipient surface also varied with the surface type and the condition (wet or dry) of the virus inoculum. Recovery of virus from raspberries was lower by far than recoveries from other surfaces. Raspberries have many more crevices and indents, which unlike grapes and blueberries, may have allowed for the virus to better adhere to the fruit and become sheltered, avoiding elution. Additionally, the method of elution for raspberries differed from the method for grapes and blueberries; they were not vortexed during elution but instead were inverted repeatedly in elution buffer as they would otherwise break apart, potentially releasing compounds that would be inhibitory toward RT-PCR. All samples from recovery controls tested negative for inhibitors of RT-PCR when an internal amplification control was included (data not shown). Differences in recovery percentages between the different recipient surfaces necessitated adjustment of the raw transfer data to account for these differences so that comparisons could be made between the transfer events. Adjustment of the data in this way has been described by others in similar studies (5, 6).

MNV-1 was included as part of the lower titer virus cocktail. While MNV-1 is used as a surrogate for determining the infectivity of human NoV, no studies have adequately addressed its potential as a surrogate for human NoV transfer studies. In this study, a comparison of virus transfer among GI.1b, GII.4, and MNV-1 viruses found that MNV-1 transfer was similar when compared with that of the human NoVs used in this study. One exception to this trend was observed in scenarios of fomite transfer. MNV-1 transfer under wet conditions occurred to a lesser degree than did GI.3b or GII.4 NoV transfer. This indicates that MNV-1 is a good model for single-transfer studies, but more studies may be needed to determine whether MNV-1 can be used as a surrogate for human NoV in multiple-transfer studies.

The virus stock for the high-titer GII.4 experiments was a 20% fecal suspension, while the experiments using a lower titer virus cocktail contained a mixed medium that included the 20% fecal suspensions from GI and GII clinical specimens and clarified cell culture lysate remaining after ultracentrifugation and resuspension of MNV-1. D'Souza et al. (13) examined transfer of human NoV suspended in clarified fecal material and transfer of FCV suspended in clarified cell culture lysate. Bidawid et al. (6) determined transfer rates for FCV suspended in Earl's balanced salt solution containing bovine serum albumin, mucins, and

tryptone to simulate a fecal matrix, and in a previous study by this group, transfer was determined for hepatitis A virus suspended in a solution containing cell culture lysate and 5% FBS to simulate organic soil load (5). In this study, the impact of the inoculum matrix on virus transfer was investigated. Virus transfer from gloved fingertips to stainless steel was compared for three inoculation matrices, as follows: (i) ultracentrifuged virus stock resuspended in PBS containing 5% FBS, (ii) ultracentrifuged virus stock resuspended in a 10% fecal suspension that tested negative for GI and GII NoV, and (iii) an unaltered virus stock which was similar to the three-virus cocktail used in the majority of this study. Differences were observed between the virus stocks, indicating that the suspending matrix had an impact on virus transfer. However, there was no difference between transfer of the three viruses, further supporting the use of MNV-1 as a surrogate for this type of transfer study. In this set of experiments, the transfer rates of the viruses in the unaltered virus stock were higher than those of the low-titer cocktail. A new virus cocktail had to be prepared for this set of experiments as an insufficient amount of fecal material was available from the GI.3b and GII.4 NoV stool suspensions used in previous studies. Differences in transfer percentages are likely the result of virus preparation.

A limitation of this study is that the interaction between the pathogens and gloves may not be the same as between the pathogens and human skin. Differences between latex gloves and human skin include surface charge, surface texture, and the presence of oils on the skin, all of which could affect the amount of virus reduced after treatment or the amount of virus transferred between surfaces. Important questions regarding the differences between transfer under wet and dry conditions, differences in transfer between single objects and multiple objects, the use of MNV-1 as a surrogate for such studies, and the impact of the inoculum matrix were all addressed in the current study. It is likely that several of these findings will be similar in bare-hand-contact studies, but such studies should be conducted for verification.

For the high-titer GII.4 transfer studies, 10- μ l amounts of virus stock containing 10^9 genomic copy numbers of virus were inoculated onto donor surfaces. The high-titer GII.4 stock used in this study was derived from a clinical specimen containing 10^{11} genomic copy numbers per g of fecal material. While this is a very high virus concentration, similar concentrations have been observed previously (1). This set of experiments is representative of a worst-case scenario. More realistic levels of contamination are demonstrated in transfer studies, with the lower titer cocktail starting with 10^6 genomic copy numbers of each virus in each 10- μ l inoculum. A person shedding 10^8 viruses per g of feces may have similar levels of virus in 10 μ l of fecal material. Both symptomatic and asymptomatic individuals can shed virus at this concentration (1).

The results of the virus transfer study are important because they represent the first quantitative study for human and surrogate NoV transfer which can be used in a quantitative risk assessment model. Overall, there was not a significant difference between GI.3b, GII.4, and the

MNV-1 surrogate during transfer, indicating that MNV-1 is a suitable surrogate for transfer studies. In conclusion, the NoV transfer data support the idea that there is risk that an individual shedding NoV who uses the restroom and contaminates his or her hands, without washing, will further contaminate both restroom door handles and produce, such as small fruits. Additionally, a healthy person who uses the restroom may wash their hands but contaminate them upon exiting and proceed to contaminate produce. While contamination of hands with 10 μ l of fecal material represents a worst-case scenario, the data presented here show one method by which people may potentially cause a NoV outbreak during food handling, emphasizing the importance of good hand hygiene.

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