Comparison of the Activity of Alcohol-Based Handrubs Against Human Noroviruses Using the Fingerpad Method and Quantitative Real-Time PCR

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Abstract Noroviruses (NoV) are the most common cause of acute nonbacterial gastroenteritis in the United States, and human hands play an important role in their transmission. Little is known about the efficacy of hand hygiene agents against these highly infectious pathogens. We investigated the activity of seven commercially available hand hygiene products against human noroviruses by in vivo fingerpad tests. The in vivo activity of alcohol-based handrubs ranged from 0.10 to 3.74 log reduction and was not solely dependent on alcohol concentration. A handrub (VF481) based on 70% ethanol and a blend of other skin care ingredients reduced Norwalk virus (NV) by 3.74 log in 15 s and provided significantly greater NV reduction than all the other products tested (P < 0.001). Furthermore, VF481 was the most effective product tested against the NoV genogroup II strains Snow Mountain virus (GII.2) and a GII.4 strain. These results demonstrate that alcohol by itself is not effective against NoV, but effective formulation of alcohol-based handrubs can achieve significant reduction of norovirus RNA on fingers.

Keywords Norovirus · Quantitative RT-PCR · Handrub · Fingerpad · ASTM

Introduction

Noroviruses (NoV) are the major cause of acute nonbacterial gastroenteritis in humans worldwide (Widdowson et al. 2005; Blanton et al. 2006; Lopman et al. 2002). In the United States, NoV account for 59% of the estimated 76 million food-related infections (Mead et al. 1999) and have also been implicated in outbreaks in both long-term care and acute care facilities (Wu et al. 2005; Hansen et al. 2007). Human NoV are classified into three genogroups, designated GI, GII, and GIV, and multiple clusters (genotypes) within each genogroup. Norwalk virus (NV) and Snow Mountain virus (SMV) are prototype GI.1 and GII.2 genotypes, respectively. GII.4 is the most commonly detected NoV genotype throughout the world and accounted for 62% of all reported NoV outbreaks from 2001 to 2007 (Siebenga et al. 2009).

Hands are known to be an important vehicle for the transmission of NoV (Bidawid et al. 2004; Todd et al. 2008; Moe et al. 2001), and the use of hand hygiene products that are effective at removing or inactivating NoV is likely to be a critical part of an effective NoV infection control strategy (Bidawid et al. 2004; Lages et al. 2008; Kampf et al. 2005). Despite the important role of hands in
the transmission of NoV, little is known about the efficacy of hand hygiene practices in reducing the spread of NoV.

Alcohol-based handrubs have some advantages over traditional hand washes, particularly when running water is not accessible. The effectiveness of handrubs against bacteria (Davis et al. 2006; Kampf et al. 2003; Weber et al. 2003), and viruses, including influenza (Kramer et al. 2006), rotavirus (Sattar et al. 2000; Sattar and Ansari 2002), hepatitis A virus (Mbithi et al. 1993), poliovirus (Mbithi et al. 1993), adenovirus (Sattar et al. 2000), and rhinovirus (Sattar et al. 2000) has been widely studied. The efficacy of handrubs against human NoV has been rarely reported primarily due to the lack of an available animal model or routine culture system for studying human NoV infectivity.

Although preliminary results from Straub et al. (2007) demonstrate that it may be possible to culture NoV, routine and replicable culture-based methods to quantify infectious human NoV are not yet available. Most studies on the efficacy of disinfectants and antiseptics against NoV have employed surrogate animal caliciviruses, including feline calicivirus (FCV) (Gehrke et al. 2004; Kampf et al. 2005) and more recently murine norovirus (MNV) (Cannon et al. 2006; Macinga et al. 2008; Park et al. 2010; Okunishi et al. 2010; Magulski et al. 2009), to predict the behavior of human norovirus. These viruses are useful because they can be measured via cell culture assays and possibly by animal challenge studies. However, the appropriateness of FCV and MNV as surrogates for human NoV has been debated because they do not always behave in the same manner in environmental persistence or inactivation studies (Park et al. 2010). Specifically, FCV is quite pH sensitive under acidic conditions, while MNV is sensitive to desiccation (Cannon et al. 2006) and is likely much more susceptible to ethanol inactivation (Belliot et al. 2008; Magulski et al. 2009; Park et al. 2010). These observations are inconsistent with current knowledge of the behavior of the human NoV. Synergistically formulated ethanol-based handrubs have recently been reported to have significantly enhanced antiviral efficacy against several non-enveloped viruses, including FCV and MNV (Macina et al. 2008; Belliot et al. 2008). Moreover, the results obtained using surrogate viruses may not be a good indication of the actual efficacy of these products against human NoV strains. It is therefore important to evaluate hand hygiene products against human NoV to determine the ability of antiseptics to reduce transmission of NoV by hands.

Quantitative real-time PCR (RT-qPCR) is currently the best available method for the enumeration of human NoV genomic copies in food, clinical, and environmental samples (Topping et al. 2008; Duizer et al. 2004; Rodriguez-Lazaro et al. 2007). Recently, we combined RT-qPCR and the ASTM fingerpad methodology (Sattar and Ansari 2002; E-1838-02, ASTM International 2002) to test hand hygiene products against Norwalk Virus (NV). Our results indicated that the performance of an antimicrobial handwash was similar to that of a water rinse, and that a handrub based on 62% ethanol was not effective at reducing NV on fingerpads (Liu et al. 2010). The aim of this study was to examine the efficacy of several commercial alcohol-based handrubs against three human NoV strains, NV (GI.1), SMV (GI.2) and a recent GI.4 isolate, using the fingerpad method and RT-qPCR assays for virus quantification. This study expands our previous work with NV by including additional human NoV strains and evaluating the efficacy of additional hand hygiene agents—including those previously shown to be effective against surrogate animal caliciviruses (Macina et al. 2008).

### Materials and Methods

#### Antimicrobial Test Products

A total of seven commercially available hand hygiene products were evaluated (Table 1): seven products against NV (Table 2 and Fig. 1), three against SMV (Table 3), and two against GI.4 (Table 4). The benchmark handrub (PURELL Original Instant Hand Sanitizer, subsequently referred to as “Benchmark”), PURELL Instant Hand Sanitizer VF447 (subsequently referred to as “VF447”), and PURELL Instant Hand Sanitizer VF481 (subsequently referred to as “VF481”) are commercially available products from GOJO Industries Inc., Akron, OH. Other products that are commercially available handrubs were purchased through standard market distributors and were all tested before their expiration dates on the label.

#### Virus Inoculum

Norwalk virus and Snow Mountain virus were obtained from the stool samples of previously healthy adult volunteers who became infected with norovirus in previous human challenge studies (Lindesmith et al. 2003, 2005). GI.4 norovirus was kindly provided by Dr. Lee-Ann Jaykus (North Carolina State University), and was confirmed by RT-PCR and sequencing a 172 bp fragment of the capsid region that showed 100% homology with 2006-USA GI.4 Minerva strain. The stool samples were diluted to 20% suspensions with RNAse-free water, vortexed briefly, and centrifuged at 550 g for 30 s. To ensure the safety of the study participants, careful screening and strict exclusion criteria for volunteer enrollment and post decontamination procedures were performed as described below.
Human Test Subjects

Study protocols involving human volunteers were reviewed and approved by the Institutional Review Board of Emory University. A total of 30 adult volunteers, between 18 and 50 years of age, were enrolled in the study. After providing informed consent, both hands of each volunteer were carefully inspected prior to each experiment to ensure that they were free of any cuts, abrasions or rashes.

Fingerpad Method

A modification of the ASTM (American Society for Testing and Materials) standard E-1838-02 fingerpad method,
which has been described in detail previously, was used in this study (Sattar and Ansari 2002; Macinga et al. 2008; E-1838-02, ASTM International 2002). The modification involved not scraping the fingers after the handrub use in order to avoid physical removal of the virus and to more accurately model actual use of a handrub by a consumer.

Prior to inoculation of the fingerpads, the volunteers were asked to clean their hands completely by washing with a mild soap followed by sanitizing with a solution of 70% ethanol.

### Inoculation of Fingerpads

Ten microliters of a 20% suspension of NV (approximately $6.3 \times 10^6$ genome copies), SMV (approximately $2.0 \times 10^9$ genome copies), or GII.4 NoV (approximately $6.4 \times 10^6$ genome copies) were placed on the center of each fingerpad of volunteers’ hands. For determination of input virus (time 0 control), the seeded inoculum was eluted immediately from the thumbs after fingerpad inoculation (without drying) with 990 μl of Hanks Balanced Salt Solution (HBSS). The thumbs were decontaminated as described below, and the virus inocula on other digits were air dried for 20 min.

### Test Method for Handrub Test Products

For all handrub test products, the fingerpads were exposed to 1 ml of the test substance in an open vial after virus inoculation and drying, respectively, for a contact time of 15 s without inversion. Upon completion of product testing, the volunteers were instructed to wash their hands with antibacterial liquid soap, and their fingerpads were then decontaminated by pressing onto a paper towel soaked with 10% bleach for 2–3 min.

### Virus Elution from Fingerpads

To elute the virus remaining on a control fingerpad after drying or on fingerpads after treatment with test products, the volunteers were asked to place the contaminated area of the fingerpad over the mouth of a 1.7 ml plastic vial containing 1 ml of HBSS. The vial was inverted and the eluent in the vial was allowed to remain in contact with the inoculated area for 10 s. The vial was next inverted 20 times with the fingerpad still in place. The soak and inversion steps were repeated once. The vial was then turned upright, and the eluate remaining in contact with the fingerpad was scraped against the inside rim of the vial to recover as much of the fluid as possible.

### Virological Analysis

#### Recovery of Viruses from Eluates

The viruses in the eluates were precipitated by adding 12 mg polyethylene glycol (PEG) 8000 (Sigma, St. Louis, MO), incubated at 4°C for 1 h, and centrifuged at 12,000×g for 15 min. The virus-containing pellets were reconstituted in 50 μl of RNAse-free water prior to freezing at −80°C.

#### RNA Extraction and Real-time RT-PCR

For release of RNA, a heat release RNA extraction was used as described previously (Schwab et al. 1997) for all experiments except for those presented in Fig. 1. An alternative RNA extraction method, the QIAamp Viral RNA kit (QIAGEN, Valencia, Calif.), was used in accordance with the manufacturer’s instructions for the experiments in Fig. 1. NV specific RT-qPCR (genogroup I) that targets the RNA-dependent RNA polymerase region of the NV was carried out following methods described previously (Teunis et al. 2008). SMV and GII.4 RNA were quantified using a norovirus GII broadly reactive RT-qPCR assay that the primers and probe span the open reading frame 1 (ORF1) and ORF2 junction region (Kageyama et al. 2003). To generate a standard curve for NV RNA quantification, a full-length NV RNA standard was in vitro transcribed from NV plasmid cDNA with T7 RNA polymerase (Ambion Inc.), serially diluted and quantified by UV absorbance at 260 nm. Similarly, a SMV RNA standard was generated from SMV plasmid with a 2179-bp insert (nt 3,000–5,178) spanning the entire RNA-dependent
RNA. In this study, we examined the efficacy of six hand hygiene products against Norwalk virus (Table 2). There were considerable differences in the viral RNA reductions by the different hand hygiene products, with mean log reductions from 12 replicate fingerpads (6 fingerpads for Anios Gel 85 NPC) ranging from 0.10 to 3.74. Exposure to VF481 gave the greatest mean reduction of NV RNA (3.74 log) that was significantly higher than any other product tested ($P < 0.001$). Sanitizers VF447, Endure 300 and Anios Gel 85 NPC, provided moderate NV RNA reduction (1.27–2.04 log) and were significantly different than the baseline controls ($P < 0.001$). The lowest NV RNA reductions were by Sterillium Virugard and Germstar Noro and were not significantly different from the baseline control ($P > 0.05$).

**NoV Strains Exhibit Unique Sensitivities to Alcohol-Based Handrubs**

To investigate potential strain-to-strain differences in NoV reduction by handrubs, we evaluated the efficacy of the two most effective hand hygiene products in our first experiments (VF481 and VF447) and the benchmark handrub product against SMV in a trial with six subjects. VF481 was again the most effective product, reducing SMV RNA by a mean 2.27 log reduction using heat release RNA extraction method (Table 3). However, SMV RNA reduction was less than that observed for VF481 against NV RNA (3.74 log reduction) in the previous trial in Table 2. The SMV RNA reduction by VF481 was significantly different from the baseline control samples ($P < 0.0001$) and also from the RNA reductions associated with the benchmark sanitizer ($P = 0.0055$) and VF447 ($P < 0.0001$).

The performance of the benchmark sanitizer and VF481 were also compared against NoV GII.4 using heat release RNA extraction method (Table 4). VF481 reduced the GII.4 RNA by a mean of 4.02 log in a 15-s exposure in comparison to 2.30 log for the benchmark sanitizer at the same exposure time ($P < 0.0001$).

**Impact of RNA Extraction Methods on Estimates of NoV Reduction**

Two RNA extraction methods, heat release and Qiagen, were compared for NV and GII.4 NoV to determine if the RNA reductions we observed were due to PCR inhibition rather than virus inactivation. Two products were examined in this experiment—the 62% ethanol “benchmark” handrub (Liu et al. 2010) and VF481. For both test products, the mean NoV RNA reductions were lower in samples processed by the Qiagen method (1.88 log for VF481 and 0.29 log for benchmark against NV) compared to the reductions measured in aliquots of the same samples processed by the heat release method (3.70 log for VF481 and...
0.92 log for the benchmark sanitizer against NV) (Fig. 1, left). This difference was significant ($P < 0.0001$) for VF481 but not significant ($P = 0.09$) for the benchmark sanitizer. Similar trends were observed for the GII.4 virus (Fig. 1, right) when comparing the Qiagen and heat release results ($P = 0.01$ for VF481, and $P = 0.25$ for the benchmark). These findings suggest that the NoV RNA reductions observed for some test products (VF481 and possibly others) using the heat release method may be overestimated due to PCR inhibitors even though the samples were tested at a 1:100 dilution. Despite these differences, both extraction methods indicated similar trends in terms of the relative magnitude of RNA reduction by each product for the two different NoV strains. For both extraction methods and for both virus strains, VF481 produced significantly greater mean NoV RNA reductions than the benchmark sanitizer ($P < 0.0001$).

### Discussion

In this study, we examined the efficacy of seven commercial hand hygiene products against multiple NoV strains using the fingerpad method and RT-qPCR. A wide range of efficacy (between 0.10 and 3.74 log reduction) was observed. The most effective product tested, VF481, is a 70% ethanol gel containing additional ingredients that appear to potentiate the virucidal activity of the product. The mean NoV RNA log reductions produced by VF481 ranged from 2.27 for SMV (Table 3) to 3.74 for NV (Table 2) and 4.02 for GII.4 (Table 4) using the heat release method. The efficacy of the other five commercial products (with ethanol concentrations from 62% to 95% [see Table 1]) against NV ranged from 0.10 to 2.04 log reduction (Table 2). These results indicate that formulation plays an important role in product efficacy and that alcohol alone does not dictate NoV reduction. In products like VF447 and VF481, additional ingredients, such as citric acid, polyquaternium-37 or copper gluconate (Table 1), may work with the ethanol to help denature the viral capsid protein. Further studies are needed to specifically examine the effect of these additives on viral RNA and clarify our understanding of the mechanism of action. However, the increased magnitude or broader spectrum of virucidal activity from these synergistic blends has been reported in two previous studies using norovirus animal surrogates as well as poliovirus, rotavirus, adenovirus, hepatitis A virus, and bacteriophage MS2 (Kramer et al. 2006; Macinga et al. 2008). Mean MNV log reduction (measured by plaque assay) was 1.16 for the benchmark sanitizer vs. $\geq 3.68$ for VF447 in in vitro studies and 0.91 for 75% ethanol vs. 2.48 for VF447 in fingerpad studies (Macinga et al. 2008).

Because NoV strains are highly diverse, handrubs need to be effective against a range of NoV strains including the predominant circulating epidemiological NoV strains. Our results indicate differences in NoV reduction depending on the virus strain and demonstrate the importance of testing more than one strain. The GII.4 NoV was more readily reduced from fingerpads than NV by both VF481 and the benchmark sanitizer (Fig. 1). This finding is consistent with previous research (Butot et al. 2008, 2009) but is somewhat surprising because GII.4 NoV strains have been the predominant NoV outbreaks strains for years (Siebenga et al. 2009), therefore we expected they might be more resistant to inactivation on hands. In contrast, the SMV strain appeared to be more resistant than NV to the handrubs tested but was still significantly reduced by VF481 (Table 3). These findings suggest that VF481 could be helpful in controlling outbreaks due to various NoV strains. Further evaluation of other strains would be of value.

This study extends our previous work and knowledge (Liu et al. 2010) in two ways. First, it demonstrates that some alcohol-based handrubs can be effective against human NoV as measured by RNA reduction using RT-qPCR, and that there are significant differences in the ability of various hand hygiene products to reduce these viruses on fingerpads. These findings highlight the need for evidence-based decision-making about hand hygiene products in settings where NoV transmission and outbreaks can occur. Second, different human NoV strains display different susceptibilities to hand hygiene agents, so it is important to evaluate these products against several NoV strains.

Several questions arise because the methodology in this study uses the presence of RNA genome copies as an indicator of infectious virions. However, it is possible that naked RNA from inactivated virus may be detected by RT-PCR in the fingerpad eluates—thus resulting in an underestimate of the efficacy of the hand hygiene agent. We addressed this concern in our previous study where we compared samples pre-treated with RNAse H to duplicate untreated samples and found no significant difference between the results (Liu et al. 2010). However, some investigators have reported differences in the log reductions of surrogate animal caliciviruses measured by RT-PCR and by plaque assay (Belliot et al. 2008; Park et al. 2010). Therefore, reduction in measurable viral RNA should be considered a conservative measure of the efficacy of a hand hygiene product, and side-by-side comparisons with culture methods have confirmed that significant reductions in RNA titer consistently reflect significant reductions in infectivity of MNV (Park et al. 2010).
Another potential concern is that the observed virus reduction may have been overestimated due to PCR inhibitors in the test products. A side-by-side comparison of the RNA heat release method and the Qiagen RNA extraction method, which is designed to remove PCR inhibitors, showed less NoV RNA reduction in samples processed by the Qiagen method than the same samples processed by the heat release method. For the benchmark sanitizer, there was not a significant difference between the measured reductions from the two RNA extraction methods. However, for VF481, the PCR titer of NoV RNA was significantly lower in the samples tested by the heat release method and may be due in part to residual PCR inhibitors in these samples even though they were tested at a 1:100 dilution. These results suggest that the heat release results for all the test products in this study may represent the upper bound of NoV reduction by these products. Future method developments, such as using immunomagnetic separation (IMS) assay to detect RNA from virions with intact capsids and developing an internal RNA control to directly control for the presence of PCR inhibitors is under investigation.

The true measure of efficacy of a hand hygiene product is the impact on NoV outbreak control or prevention in high-risk settings where NoV outbreaks commonly occur, such as food service and food processing, healthcare (acute care hospitals and long-term elder care facilities), cruise ships and military vessels. Is the 2–4 log NoV reduction associated with VF481 sufficient to significantly reduce or prevent NoV transmission via hands? One recent report indicates that a NoV outbreak in an infirmary in Hong Kong was successfully contained by directly observed hand hygiene with WHO formulation of a handrub with 80% vol/vol ethanol (Cheng et al. 2009). Further field studies are needed with products that have demonstrated efficacy against human noroviruses.

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