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New *In Situ* Capture Quantitative (Real-Time) Reverse Transcription-PCR Method as an Alternative Approach for Determining Inactivation of Tulane Virus

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Human noroviruses (HuNoVs) are the major cause of epidemic nonbacterial gastroenteritis. Although quantitative (real-time) reverse transcription-PCR (qRT-PCR) is widely used for detecting HuNoVs, it only detects the presence of viral RNA and does not indicate viral infectivity. Human blood group antigens (HBGAs) have been identified as receptors/co-receptors for both HuNoVs and Tulane virus (TV) and are crucial for viral infection. We propose that viral infectivity can be evaluated with a molecular assay based on receptor-captured viruses. In this study, we employed TV as an HuNoV surrogate to validate the HBGA-based capture qRT-PCR method against the 50% tissue culture infectious dose ($TCID_{50}$) method. We employed type B HBGA on an immuno-well module to concentrate TV, followed by amplification of the captured viral genome by *in situ* qRT-PCR. We first demonstrated that this *in situ* capture qRT-PCR (ISC-qRT-PCR) method could effectively concentrate and detect TV. We then treated TV under either partial or full inactivation conditions and measured the remaining infectivity by ISC-qRT-PCR and a tissue culture-based amplification method ($TCID_{50}$). We found that the ISC-qRT-PCR method could be used to evaluate virus inactivation deriving from damage to the capsid and study interactions between the capsid and viral receptor. Heat, chlorine, and ethanol treatment primarily affect the capsid structure, which in turns affects the ability of the capsid to bind to viral receptors. Inactivation of the virus by these methods could be reflected by the ISC-qRT-PCR method and confirmed by $TCID_{50}$ assay. However, the loss of the infectivity caused by damage to the viral genome (such as that from UV irradiation) could not be effectively reflected by this method. Despite this limitation, the ISC-qRT-PCR provides an alternative approach to determine inactivation of Tulane virus. A particular advantage of the ISC-qRT-PCR method is that it is also a faster and easier method to effectively recover and detect the viruses, as there is no need to extract viral RNA or to transfer the captured virus from magnetic beads to PCR tubes for further amplification. Therefore, ISC-qRT-PCR can be easily adapted for use in automated systems for multiple samples.

Human noroviruses (HuNoVs) are highly contagious viruses that are the primary cause of acute gastroenteritis, and they account for approximately 60% of food-borne illnesses in the United States (1). Its routes of infection include person to person, contact with contaminated surfaces, and ingestion of contaminated food/water. HuNoVs are pathogens of significant concern, but as of now they cannot be cultured, which limits the approaches to detect and study them to molecular methods. Molecular method-based virus quantitation assays, notably quantitative (real-time) reverse transcription-PCR (qRT-PCR), have been used in lieu of *in vivo*/*ex vivo* assays for a number of reasons, including speed, ease, and feasibility. It is, however, questionable whether what they quantitate is an accurate reflection of viable viruses. A virus could lose its infectivity by damage to its viral capsid, but the viral RNA may still persist to be detected by qRT-PCR. Similarly, a treatment can lethally damage the viral RNA at one or more regions outside the qRT-PCR amplicon, yet the amplicon may still persist to be detected by qRT-PCR. Although qRT-PCR is the most sensitive method, it has been a challenge to distinguish infectious HuNoV from inactivated HuNoV among positive testing samples. Prior deactivation assays using Tulane virus (TV), a culturable analogue of HuNoV, have shown that there is little to no correlation between the virus counts obtained from 50% tissue culture infectious dose ($TCID_{50}$) assays and the virus signal obtained from traditional qRT-PCR assays (2). While qRT-PCR is effective at quantitating the integrity of the targeted amplicon, it cannot detect damage to other areas of the viral genome outside the qRT-PCR target amplicon; most notably, it cannot detect critical degradation of the capsid that is required for binding and infection. Therefore, qRT-PCR is only capable of detecting a subset of deactivating injuries/degradations to the virus, and its quantitation is inflated by false positives from otherwise deactivated/degraded virions.

Virus-like particles (VLP) have been used for studies on structure and immunogenicity. Lou et al. demonstrated high-pressure processing (HPP) treatment could disrupt the capsids from HuNoV, murine norovirus (MNV), and feline calicivirus (FCV) (3). However, the VLP capsid of HuNoV is highly resistant to HPP relative to its surrogates. Feng et al. reported on the inactivation of an HuNoV surrogate (MNV) by gamma irradiation as measured by tissue culture and characterization of the changes in antigenic-

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ity, capsid protein, and capsid structure on MNV and HuNoV VLP by gamma irradiation, as measured by SDS-PAGE, Western blotting, and electron microscopy (EM) (4). Regardless, VLP does not contain any viral genome, and the sensitivity of the VLP-based assay is too low. A new technique pioneered by our group involves isolating viruses using their functional receptors, followed by qRT-PCR (5). As human blood group antigens (HBGAs) have been identified as receptors/coreceptors for HuNoV, biologically isolated HBGAs (human saliva or porcine gastric mucin) and synthetic oligosaccharides have been used for conjugation to magnetic beads to concentrate HuNoV from food and environmental samples (5–9). Drawbacks of the magnetic bead method include significant bead (and presumably virus) loss during the collection process and the necessity of transferring released viral RNA into another tube for qRT-PCR. In this study, we modified the HBGA-conjugated magnetic bead method toward increased convenience and suitability for automation. Nunc Top Yield Immuno well strip modules (VWR, Brisbane, CA) were used for the assay. The immunostrip modules were originally introduced by Sano et al. (10) for immuno-PCR assays to be performed in common thermal cyclers, but they could also be coated with specific polyclonal antibodies against corresponding pathogens or biological markers with significantly improved sensitivity (11). This enzyme-linked immunosorbent assay (ELISA)/PCR hybrid reaction on the surface of a well replaces the role of the magnetic beads both as a means to capture and isolate viruses from solution and as the container in which to amplify the captured and isolated viral RNA *in situ* by qRT-PCR. By eliminating the need to extract viral RNA as a separate process, as well as the need to transfer the viral RNA to enable subsequent amplification, the HBGA-based *in situ* capture qRT-PCR (ISC-qRT-PCR) method is a faster and easier means to isolate and detect norovirus and its surrogates.

We have previously proposed that the HBGA-based capture assay be used as a means to distinguish infectious virus from inactivated virus (5). Recently, receptor binding-based assays have been explored for inactivation studies. Dancho et al. demonstrated that the viral RNA signals derived from HuNoV captured by PGM-conjugated magnetic beads were significantly reduced when HuNoV was treated by HPP and by heat inactivation (12). However, the results measured by this HBGA-conjugated capture qRT-PCR method for HuNoV could not be confirmed by tissue culture-based infectivity assays. Li et al. attempted to validate this method using the MNV system. Cell line RAW 264.7 and ganglioside GD1a were used as binding receptors in combination with RT-PCR to evaluate the inactivation status of MNV (13). There was no correlation between the inactivation of MNV as measured by a tissue culture-based assay (plaque assay) and either cellular receptor-based or ganglioside-based capture qRT-PCR (13). Being that HuNoV utilizes HBGAs as cellular receptors for infection (14) while MNV uses sialic acid as a functional receptor (15, 16), MNV is not an ideal surrogate to simulate HuNoV for the purpose of receptor binding studies. Unlike the other surrogates, yet similar to HuNoV, the recently discovered TV recognizes type A and type B HBGAs as receptors for infection (17) and may serve as an improved surrogate for HuNoV for the purpose of receptor binding studies. We used TV as a surrogate for HuNoV in this study to explore if ISC-qRT-PCR could be used as an alternative method to distinguish infectivity of the virus by comparing it side by side to tissue culture-based assays.

MATERIALS AND METHODS

Virus cultivation. LLC-MK2 cells (American Type Culture Collection, Manassas, VA) were used for culturing and titration of virus (18). Tulane virus was kindly provided by Xi Jiang (Division of Infectious Diseases, Cincinnati Children's Hospital Medical Center, Cincinnati, OH). Cells were grown in HyClone M199/Earle balanced salt solution (EBSS) medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 2× Gibco antibiotic-antimycotic (Life Technologies, Carlsbad, CA). One day after plating cells, confluent cell cultures were inoculated with virus at a multiplicity of infection (MOI) of 0.1 and then harvested by scraping at 2 days postinoculation. The cultures were then transferred to sterile 50-ml centrifuge tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and freeze-thawed (alternating cycles of −20°C and 25°C) three times to release cell-associated viruses. Cellular debris was removed by centrifugation at 2,500 relative centrifugal force (RCF) for 10 min. The virus-enriched supernatant was aliquoted and stored at −20°C.

Determining virus titers by $TCID_{50}$ assay. The virus stock was used for the 50% tissue culture infective dose ($TCID_{50}$) assay (2). One hundred microliters of serially diluted virus stock, ranging from 10^{-1} to 10^{-5}, was added to each quantization well in a 96-well plate, organized with each serial dilution occupying a row, at 10 quantization wells/row. The plate was incubated for 5 to 6 days and then examined for cytopathic effect (CPE) via microscopy. CPE counts were converted into $TCID_{50}$ and $TCID_{50}$/ml using a Reed and Muench calculation method (http://www.urmc.rochester.edu/mbi/resources/xenopus/protocols/tcid50-protocol.pdf). Similarly, viable virus counts after treatment with experimental conditions (described above) were also determined by $TCID_{50}$ assay.

ISC-qRT-PCR. Human saliva was collected from three blood type B volunteers and mixed. The sample was processed immediately after collection. No personal information was collected and used. The study was approved by the Institutional Biosafety Committees (IBC) of the College of Agriculture and Biology, Shanghai Jiao Tong University. The aggregated saliva was boiled for 5 min and then centrifuged at 10,000 RCF for another 5 min. The clarified supernatant was aliquoted and stored at −20°C. The wells of the Nunc Top Yield Immuno module strip (VWR, Brisbane, CA), which was designed to perfectly fit into the module well of the commonly used qRT-PCR machines, were coated with 100 μl of a 1:1,000 dilution (using 0.5 M carbonate-bicarbonate buffer, pH 9.6) of clarified saliva at 4°C overnight and then blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 37°C for 1 h. The wells were washed with PBS and used immediately. One hundred microliters of TV was added to each well, incubated at 37°C for 30 min, and then washed three times with PBS to clear any unbound viruses. Ten microliters of RNase-free distilled water (dH_2O) was added to each well, which were then sealed with polyolefin sealing film (VWR, West Chester, PA), brought to 95°C for 5 min, and cooled to 4°C. To this heat-released RNA in solution, 12.5 μl of a prepared qRT-PCR master mix from the one-step qRT-PCR kit (Quantitect probe RT-PCR kit; Qiagen, Valencia, CA) was added, and the wells were then resealed with polyolefin sealing film. The primers were the same as those described by Kageyama et al. (19), with different fluorophores and quenchers (Integrated DNA Technologies, Inc., San Diego, CA). The primers for qRT-PCR were located at the RNA-dependent RNA polymerase region in open reading frame 1 at nucleotides (nt) 3704 to 3777, and the probe was located at nt 3731 to 3752. The sequences of primers used for detection of TV were the following: TV forward, 5′ TGA CGA TGA CCT TGC GTG 3′; TV reverse, 5′ TGG GAT TCA ACC ATG ATA CAG TC 3′; TV probe, 5′ hexachlorofluorescein (HEX)-ACC CCA AAG CCC CAG AGT TGA T-black hole quencher 1 3′. The qRT-PCR master mix contained qRT-PCR buffer and a mix of primers (300 nM), probe (100 nM), and reverse transcriptase/polymerase (1.0 U). The viral RNA was quantitated with probe-based quantitative real-time RT-PCR using a qPCR system (MX3000P; Stratagene, La Jolla, CA). Cycling times and temperatures were 50°C for 30 min and 95°C for 15

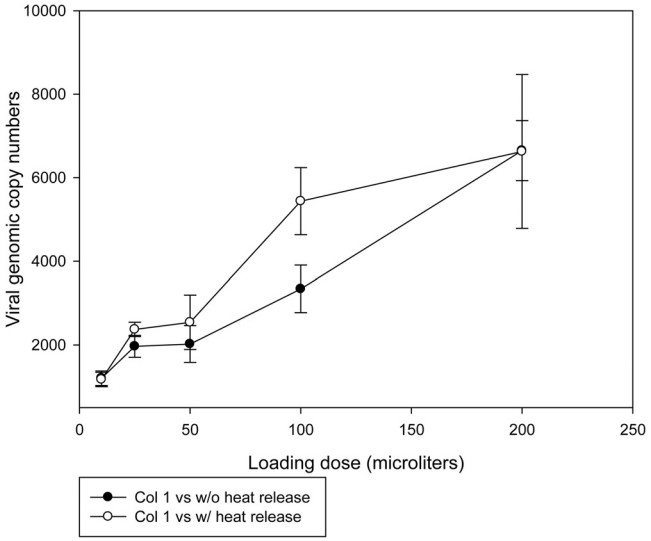

FIG 1 Recovery of TV with various loading doses and with or without heat release of viral RNA. TV copy numbers in $\log_{10}$ are indicated on the *y* axis, and loading doses are indicted on the *x* axis.

min, followed by 42 cycles of 95°C for 15 s, 53°C for 20 s, and 60°C for 50 s. A p289/p290-based TV amplicon in a TOPO vector was used to generate a standard curve (2). The TV amplicon (307 bp) was located in the RNA-dependent RNA polymerase gene of caliciviruses (2), ranging from nt 3609 to 3915. The plasmid DNA was serially diluted to a theoretical range of 10^7 to 10^{-1} copies, with a detection limit of 0.6 genomic copies (2).

Partial and full inactivation conditions for TV. (i) Thermal inactivation. Three-hundred-microliter aliquots of virus stock in 1.5-ml microcentrifuge tubes were incubated in 56°C and 72°C heat blocks for 2 min and quickly cooled on ice. The control samples were kept at room temperature.

(ii) Chlorine inactivation. Bleach (Pure Bright; Concord, Ontario, Canada) was diluted to make fresh chlorine stock. The neutralizer reagent, sodium thiosulfate (Fisher Scientific, Waltham, MA), was also made fresh at the same concentration. Three-hundred-microliter aliquots of virus stock in 1.5-ml microcentrifuge tubes were brought to experimental free chlorine conditions (300 or 600 ppm) and allowed to incubate for 10 min at room temperature. The free chlorine treatment conditions were neutralized by the addition of an equal amount (relative to the chlorine solution added) of sodium thiosulfate solution. Nontreatment, control reactions using equivalent amounts of preneutralized chlorine stock were set up in parallel.

(iii) Ethanol inactivation. Three-hundred-microliter aliquots of virus stock in 1.5-ml microcentrifuge tubes were diluted with 100% ethanol to arrive at ethanol treatment concentrations of 40% and 70% and allowed to incubate for 20 s at room temperature. The incubation is immediately quenched afterwards by a >10-fold dilution with minimal essential medium (MEM) to lower the ethanol concentration and to reduce cytotoxicity. Aliquots of virus were also diluted with MEM at corresponding concentrations (40% and 70%) for untreated controls.

(iv) UV irradiation. Three-hundred-microliter aliquots of virus stock were placed at the center of 90-mm petri dishes and placed at the center of a Stratagene UV cross-linker (Stratalinker 1800; emits UV-C at 254 nm and ~3 mW/cm^2) with a distance to the middle of the UV lamps of 16 cm. The virus was then UV irradiated with energies of 30 mJ/cm^2 (7 s) and 60 mJ/cm^2 (14 s). Nontreatment, control reactions were set up in parallel without UV irradiation.

ELISAs. Mouse anti-TV antibodies (kindly provided by Xi Jiang) were used in a sandwich ELISA similar to one described previously (20). Briefly, Nunc Top Yield Immuno module (VWR, Brisbane, CA) wells were coated with a 1:1,000 dilution of combined salivas from blood type B individuals (in 0.5 M carbonate-bicarbonate buffer, pH 9.6), incubated at 4°C overnight, and then blocked with BSA (1.0% in PBS) at 37°C for 1 h. One hundred microliters of treated or untreated TV was added to each well and incubated at 37°C for 30 min. After three washes with PBS, anti-recombinant TV (rTV) antibody (diluted 1:3,000 in PBS, 100 μl/well) was added to TV-bound wells and incubated at 37°C for 30 min. After three washes with PBS, the bound anti-rTV antibodies were detected by the addition of alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG antibodies (diluted 1:3,000 in PBS, 100 μl/well; Zymed Laboratories, South San Francisco, CA), followed by development with *p*-nitrophenyl phosphate substrate (1 mg/ml, 100 μl/well). Negative controls included PGM-coated modules without the addition of TV and BSA-blocked modules without PGM coating but with 100 μl/well of TV. The average optical density from a set of experimental wells was divided by the average optical density of all negative-control wells to obtain a value designated P/N.

Data analysis and statistics. Each plating experiment was repeated three times ($n = 3$) as independent replicates with triplicates in each experiment ($n = 3$). One-way analysis of variance (ANOVA) was employed for data analysis.

RESULTS

Optimal conditions and sensitivity of ISC-qRT-PCR method. The inclusion of the heat denaturation step (as a means to release viral RNA from receptor-captured virus) increased the measured viral RNA signal in all virus loading doses (VLD) relative to those of parallel procedures for which the heat denaturation step was omitted, except at the VLD of 200 μl/well. Although the increase was obvious, a statistical difference was obtained only at a dose of 100 μl/well ($P = 0.021$). These results suggest that the heat denaturation step was not necessary for the ISC-qRT-PCR method, although the receptor-captured viral genomic signal (RCVGS) was subjectively perceived to be enhanced by heat treatment. A dose-dependent response was observed from 10 to 200 μl/well VLD (Fig. 1). A significant increase in RCVGS was observed from 10 to 100 μl/well VLD ($P < 0.05$) in both heat-released and nontreated groups. Although the RCVGS from the VLD of 200 μl/well (6,629 ± 1,844) was higher than what obtained from the VLD of 100 μl/well (5,438 ± 803) in the heat-released group, the increase was limited and was not significantly different ($P = 0.363$). Therefore, the VLD for the remaining experiments in this study was set to 100 μl/well, and all samples were heat denatured prior to qRT-PCR for the rest of this study. The efficiency and detection limit of the ISC-qRT-PCR method were compared to those of qRT-PCR of viral RNA extracted with a commercial RNA extraction kit (Table 1). Although the signal from the ISC-qRT-PCR method was

TABLE 1 Recovery of TV, as measured by qRT-PCR of extracted viral RNA or by ISC-qRT-PCR

TV titer[a] ($TCID_{50}$ per reaction)	Measurement (copies per reaction) by:	
	qRT-PCR with extracted viral RNA	ISC-qRT-PCR
2.57×10^3	2,199 ± 191	1,083 ± 291
2.57×10^2	292 ± 33	144 ± 15
2.57×10^1	41 ± 4	26 ± 2
2.57	<0.6	<0.6
0.257	<0.6	<0.6

[a] The TV titer was 2.57×10^5 $TCID_{50}$ per ml. One hundred microliters of TV (2.57×10^4 $TCID_{50}$) was used to extract the viral RNA. The extracted viral genomic RNA was eluted by 50 μl elution buffer, and 5 μl was used as the template for qRT-PCR assay (2.57×10^3 $TCID_{50}$).

TABLE 2 Inactivation of TV as measured by ISC-qRT-PCR and $TCID_{50}$

Treatment	Copy no. (% reduction) by ISC-qRT-PCR[a]	$TCID_{50}$/well (% reduction)
Temperature (°C)		
56	555 (97.6)	4,060 (84.2)
72	<0.6 (100)	0 (100)
Chlorine (ppm)		
300	148 (99.4)	70.4 (99.7)
600	<0.6 (100)	0 (100)
UV (mJ/cm^2)		
30	9,873 (57.6)	51.8 (99.8)
60	6,635 (71.5)	0 (100)
EtOH (%)		
40	1,215 (94.8)	4,660 (81.9)
70	<0.6 (100)	0 (100)
Positive control	23,285	25,700

[a] Copy numbers were calculated from RCVGSs.

slightly lower than those from qRT-PCR of kit-extracted viral RNA ($P < 0.05$), the difference was less than 1-fold and the trade-off acceptable for the savings in work and time. The detection limit of both assays was the same (2.5 for $TCID_{50}$ or 0.6 for genomic copies).

Inactivation of TV measured by ISC-qRT-PCR and $TCID_{50}$ assay. The infectivity of TV under partial and full inactivation conditions was measured by two methods (Table 2). ISC-qRT-PCR quantitation of TV partially and fully inactivated with heat, chlorine, and ethanol treatments could be correlated to the quantitation results of $TCID_{50}$ assays. After treatment of TV with heat, chlorine, or ethanol under full inactivation conditions, neither the RCVGS measured by the ISC-qRT-PCR method nor amplification of virus measured by the $TCID_{50}$ method was detectable. After treatment of TV with heat under partial inactivation conditions, the reductions in the RCVGS or virus titer were 97.6% and 84.2% as measured by ISC-qRT-PCR and $TCID_{50}$, respectively. After treatment of TV with chlorine under partial inactivation conditions, the reductions in the RCVGS or virus titer were 99.4% and 99.7% as measured by ISC-qRT-PCR and $TCID_{50}$, respectively. After treatment of TV with 40% ethanol, the reductions in the RCVGS or virus titer were 94.8% and 81.9% as measured by ISC-qRT-PCR and $TCID_{50}$, respectively. UV inactivation status was not accurately reflected by the ISC-qRT-PCR method. At a partial inactivation dose (30 mJ/cm^2), the reductions in the RCVGS or virus titer were 57.6% and 99.8% as measured by ISC-qRT-PCR and $TCID_{50}$, respectively. However, after treatment of TV with UV under full inactivation conditions (60 mJ/cm^2), ISC-qRT-PCR only quantified a 71.5% reduction in the RCVGS, while $TCID_{50}$ quantified a 100% reduction.

Binding of viral capsid to HBGA receptor measured by ELISA. Binding of viral capsid to HBGA was significantly reduced under partial or full inactivation conditions by heat, chlorine, or ethanol ($P < 0.05$). The results suggest that the primary mechanism of viral inactivation by these three treatments was that of alteration and/or denaturation of capsid proteins, which results in the failure of receptor binding. Heat and chlorine treatments were more effective at viral inactivation than ethanol. Unlike the other treatments, UV irradiation doses as high as 600 mJ/cm^2 resulted in no significant reduction in the ability to bind to HBGA (Fig. 2). This suggests that the mechanism of viral inactivation by UV irradiation is not at the level of interaction between virus capsid and its receptor.

DISCUSSION

The use of receptor-mediated capture qRT-PCR as a means for measuring the quantity and status of HuNoV and its surrogates has been explored with various results. Dancho et al. demonstrated that the viral RNA signals derived from viral capture by PGM-conjugated magnetic beads were significantly reduced when HuNoV was treated by HPP and by heat inactivation (12). However, in the MNV model, when both cellular receptor and the ganglioside GD1a (receptor for MNV) were tested in a receptor-mediated capture qRT-PCR assay to evaluate the inactivation status of MNV, there was no correlation between the inactivation measured by tissue culture-based assay (plaque assay) and cellular receptor-based or ganglioside-based capture qRT-PCR (13). This might simply be due to differences between the surrogates and their respective receptors. By using a cellular receptor-mediated capture qRT-PCR, we could not distinguish the inactivated TV from infectious TV (S. Xu, D. Wang, D. Yang, X. Jiang, H. Liu, and P. Tian, unpublished data). We found that free RNA present in TV stock could interfere with the accuracy of the cellular receptor-mediated capture qRT-PCR assay. For the purposes of validating an improved ISC-qRT-PCR method for potential use in the determination of inactivation conditions for HuNoV, TV is a superior surrogate, as it uses the same receptor as HuNoV does for infection. In this study, we demonstrated that there is a notable correlation between the RCVGS as measured by ISC-qRT-PCR and infectivity as measured by $TCID_{50}$ for TV inactivated by heat, chlorine, and ethanol. We further demonstrated that heat, chlo-

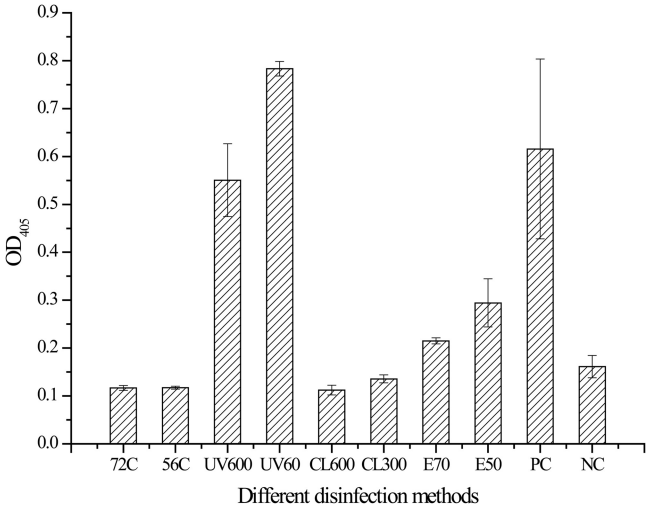

FIG 2 Binding of viral capsid to HBGA receptor measured by ELISA. The OD_{405} is indicated on the *y* axis. Inactivation conditions are indicated on the *x* axis. The temperatures 72°C and 56°C stand for treatment at 72°C and 56°C, respectively, for 2 min; UV600 and UV60 stand for treatment with UV at 600 and 60 mJ/cm^2, respectively; CL600 and CL300 stand for chlorine treatment at 600 and 300 ppm, respectively; E70 and E50 stand for treatment at 70% and 40% ethanol, respectively, for 20 s; PC and NC stand for positive and negative control, respectively.

rine, and ethanol treatment causes change primarily in the TV capsid structure and affects its ability to bind to its receptor. Being that TV and HuNoV use the same receptor for infection, we propose that the ISC-qRT-PCR method is able to provide insight into the inactivation treatments associated with capsid damage for HuNoV. We have recently demonstrated there was also a notable correlation between the reduction of captured viral genomic signal as measured by the ISC-qRT-PCR method and HuNoV inactivation deriving from capsid damage (21).

UV irradiation inactivates virus by a different mechanism. In our ELISA results, low-dose UV treatment did not reduce the ability of TV to bind to its receptor. The lack of a significant reduction in the RCVGS as measured by ISC-qRT-PCR for UV-irradiated TV samples suggests that the damage to TV is not at the capsid level. Lethal genomic damage inflicted by UV irradiation can be spread along the entire genome, and the failure of replication would be detectable by a cell culture-based assay. In contrast, if this damage does not fall within the short amplicon targeted by qRT-PCR, the amplicon will persist to produce a false positive for genomic integrity.

The ISC-qRT-PCR method could be used as an alternative method to evaluate virus inactivation resulting from capsid damage. Heat, chlorine, and ethanol treatments primarily affect the capsid structure, which in turn affects the ability of the capsid to bind to its viral receptor. Inactivation of the virus by these treatments was detected by the ISC-qRT-PCR method, and the suggested loss in infectivity was confirmed by the $TCID_{50}$ assay. However, loss of infectivity caused by damage to the viral genome (such as from UV irradiation) could not be effectively reflected by this method. To our knowledge, this is the first report to directly compare and validate the receptor-mediated capture qRT-PCR and traditional tissue culture-based assay. We have recently demonstrated using the ISC-qRT-PCR method that the RCVGS was quashed when HuNoV was treated by heat at 72°C for 4 min, by chlorine at a final concentration of 16 ppm in less than a minute, and by UV irradiation at 1 J/cm^2. However, ethanol had limited effect on high-titer samples of HuNoV (21).

The *in situ* nature of this assay both simplifies and reduces its time expenditure, as it requires neither the extraction of viral RNA nor the transfer of the released viral RNA from the immobilized magnetic beads to a separate reaction container for amplification. Therefore, ISC-qRT-PCR can be easily adapted for use in an automated system for multiple samples.

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