Title: Comparison of cell-based and PCR-based assays as methods for measuring infectivity of Tulane virus

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Running title: cell-based and PCR-based assays for Tulane virus

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Abstract.

In this study, we used Tulane virus (TV) as a surrogate for HuNoV to evaluate for correlation between two cell-based assays and three PCR-based assays. Specifically, the cell-based plaque and TCID₅₀ assays measure for infectious virus particles, while the PCR-based RNase exposure, Porcine Gastric Mucin *In-Situ*-Capture qRT-PCR (PGM-ISC-qRT-PCR), and Antibody *In-Situ*-Capture qRT-PCR (Ab-ISC-qRT-PCR) assays measure for an amplicon within encapsidated viral genome. Ten batches of viral stocks ranging from 3.41×10^5 to 6.67×10^6 plaque forming units (PFUs) were used for side by side comparison with PFU as a reference. The results indicate that one PFU was equivalent to 6.7 ± 2.3 TCID₅₀ units, 9.8 ± 10.9 RNase-untreated genomic copies (GCs), 2.8 ± 3.1 RNase-treated GCs, 0.07 ± 0.07 PGM-ISC-qRT-PCR GCs, and 0.52 ± 0.39 Ab-ISC-qRT-PCR GCs. We observed that while the cell-based assays were consistent with each other, the TCID₅₀ assay was more sensitive than the plaque assay. In contrast, the PCR-based assays were not always consistent with the cell-based assays. The very high variations in GCs as measured by both ISC-RT-qPCR assays made them impossible to correlate against the relatively small variations (<20-fold) in the PFUs or TCID₅₀ units as measured by the cell-based assays.

Keywords: Tulane virus; correlation; plaque assay; TCID₅₀ assay; ISC-qRT-PCR assay; RNase exposure assay; infectivity; correlation.

1. Introduction.

Human noroviruses (HuNoV) mainly cause acute gastroenteritis and account for approximately 60% of food-borne illnesses in the United States (Scallan et al., 2011). Although HuNoVs cause significant problems in food safety and public health, many aspects of HuNoVs are poorly understood due to the current inability to culture them. One approach to circumvent this technical issue limiting the study of HuNoV is to use a culturable surrogate, such as feline calicivirus (FCV), murine norovirus (MNV) and Tulane virus (TV). HuNoV belongs to genus *Norovirus* of the family *Caliciviridae*, which is also shared by genera *Lagovirus*, *Nebovirus*, *Sapovirus*, *Vesivirus*, *Recovirus*, and *Valovirus* (Green, 2000; Green, 2007). FCV of genus *Vesivirus* uses sialic acid on host cell surfaces as receptors (Stuart and Brown, 2007) to cause upper respiratory infection in felines (Scipioni et al., 2008; Radford et al., 2007). MNV of genus *Norovirus* also uses sialic acid as receptors for virus entry and infection of macrophages and dendritic cells (Taube et al., 2009; Wobus et al., 2004). TV of genus *Norovirus* uses both sialic acid and histo-blood group antigens (HBGAs) as receptors for infection, and replicate in the gastrointestinal tract of rhesus macaques (Farkas et al., 2010). Central to their utility, these surrogates can be cultivated in tissue culture, which allows them to be quantified for infectivity in tissue-culture-based assays.

The two most-common methods for quantifying the infectivity of HuNoV surrogates are the plaque assay, and the 50% tissue culture infective dose (TCID₅₀) assays (Cannon et al., 2006; Farkas et al., 2008; Wobus, Thackray, and Virgin, 2006). Both assays rely on the measurement of visible cytopathic effects (CPE) in cell lines that support replication of the HuNoV surrogate. The plaque assay quantifies the number of plaque forming units (PFU) in a virus

sample. TCID₅₀ is an endpoint dilution assay that quantifies the amount of virus required to produce CPE in, or kill 50% of virus-inoculated cultured cells. However, the two most-commonly-used methods of measuring the infectivity of surrogates have never been directly compared. Therefore, it has been impossible to compare results from different laboratories when different units were reported. In this study, we studied the correlation between TCID₅₀ and PFU using TV as a surrogate for HuNoV.

Molecular approaches have been developed and explored as alternative methods to identify the presence of HuNoV. These PCR-based approaches have been crucial for the study of HuNoV, and will continue to be until a protocol for culturing the virus can be developed. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and reverse-transcription polymerase chain reaction (RT-PCR) are the most commonly-used methods that have been utilized toward the intent of detecting HuNoV. However, they actually only detect for the presence of specific amplicons from the HuNoV genome, which is only suggestive of HuNoV, and does not assure that the detection target was derived from an infectious viral particle. To improve the relevance of these PCR-based viral quantitation methods, several RT-qPCR variants involving genome encapsidation have been developed. The RNase exposure assay is a variation that involves degrading free RNA from viral samples prior to viral genome extraction, leaving only capsid-protected viral genome for quantitation. Nowak et al reported that the chlorine resistance of FCV and HuNoV GII.4 could be determined using the RNase exposure assay (Nowak et al., 2011). The porcine gastric mucin coated magnetic bead (PGM-MB) assay is another variation that screens for encapsidated viral genomes possessing functional receptors prior to genome extraction. Dancho et al. reported that PGM-MB assay could be used to reflect inactivation status of HuNoV caused by high pressure treatment and chemical

sanitizers (Dancho, Chen, and Kingsley, 2012). The *in situ* capture qRT-PCR (ISC-qRT-PCR) assay is yet another variation conceptually related to the PGM-MB assay, but combines the screening medium with the qRT-PCR reaction vessel. We have previously reported that the ISC-qRT-PCR method could serve as an alternative method to evaluate the inactivation of TV and HuNoV from capsid damage (Xu et., al, 2015, Wang and Tian, 2014; Wang et al., 2014). However, the correlations between the cell-based assays and the PCR-based assays have not been studied in untreated viral stocks. In this study, using TV as surrogate for HuNoV, we evaluate the correlations between these cell-based and PCR-based assays and to determine if PCR-based assays could be used to determine the infectivity of viral stocks.

2. Materials and Methods.

2.1. Virus cultivation. Tulane virus was kindly provided by Dr. Jiang (Division of Infectious Diseases, Cincinnati Children's Hospital Medical Center, Cincinnati, OH). LLC-MK2 cells (American Type Culture Collection, Manassas, VA) were used for culturing and titration of virus (Farkas et al., 2008; Wei et al., 2008). Cells were grown in HyClone M199/EBSS medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) and 2X 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 0.2 to 1.3 in M199 media with 2% FBS, and were harvested 3-5 days post-inoculation (pi) by scraping. The cultures were then transferred to sterile 50-ml centrifuge tubes (Becton Dickinson and Company, Franklin Lakes, NJ) and freeze-thawed three times to release cell-associated viruses. Cellular debris was removed by centrifugation at 2,500 RCF for 10 min, and the virus-enriched supernatant was aliquotted to be stored at -20 °C.

2.2. Plaque assay. One-sixth of the total LLC-MK2 cells trypsinized from a T-150 flask at 100% confluency was re-suspended in 20 ml of M199 with 10% FBS. Three milliliters of cell suspension was seeded into each well of a 6-well plate, then incubated overnight until cell monolayers of 100% confluency were re-established. Media was removed from all wells, and five wells were each inoculated with 1 ml from each dilution of a viral stock serial-dilution in M199 without serum spanning the range from 10^{-2} to 10^{-6} . The remaining well was filled with 1 ml of serum free M199 media without virus to serve as a control. The plates were incubated at 37 °C and 5% CO₂ for 1 h, and gently agitated every 15 minutes. The viral dilution and media were then removed carefully, and replaced with 3 ml of M199 media containing 1% molecular-biology-grade agarose and 5% FBS. The warm agarose media was allowed to solidify before

incubating at 37 °C and 5% CO₂ for 3 days. The agarose media “pucks” were fixed with 4% formaldehyde and carefully removed. The plaques in the cell monolayer were visualized by staining with 2.3% crystal violet in 20% ethanol.

2.3. TCID₅₀ assay. Virus stock was titered using the 50% tissue culture infective dose (TCID₅₀) assay as described previously, with minor modifications (Wang and Tian, 2014). In the previous protocol, TCID₅₀ was performed in M199 without FBS. In this study, TCID₅₀ assays were performed with both M199 media without serum and M199 with 5% FBS. Plates were examined for visible cytopathic effects (i.e. cell balling) via microscopy starting from the 3rd day of incubation, through the 7th day. Balling counts were converted into TCID₅₀ and TCID₅₀/ml using the Reed & Muench Calculator spreadsheet (B.D. Lindenbach, <http://www.med.yale.edu/micropath/pdf/Infectivity%20calculator.xls>).

2.4. Viral RNA extraction and Quantitative real-time RT-PCR (qRT-PCR) assay. Viral RNA was extracted from TV samples using a commercial viral RNA extraction kit (“QIAamp RNA Viral Mini”, Qiagen USA, Valencia, CA) in accordance with the manufacturer’s protocol. The primers and probes used for detection of TV were: 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’ HEX- ACC CCA AAG CCC CAG AGT TGA T -BHQ-1 3’ (Tian et al., 2013). Extracted viral RNA was quantitated by probe-based quantitative real-time RT-PCR using a one-step qRT-PCR kit (“Quantitect Probe RT-PCR Kit”, Qiagen USA, Valencia, CA) scaled to half-reaction volumes, but otherwise in accordance with the manufacturer’s protocol. Automated thermal cycling and data acquisition was performed on a MX3000P qPCR system and MxPro software (Stratagene; La Jolla, CA), with threshold determination at default settings (Wang et al., 2014).

The viral genomic signal (VSG) was presented by Ct values and converted to genomic copies (GCs) by the standard curve as described in later section.

2.5. RNase exposure assay. One hundred units of RNase I (Life Technologies, Grand Island, NY) was added to each 150 μ L TV stocks and incubated at 37 °C for 30 min, then quickly followed by heat-inactivation of the enzyme at 95°C for 5 min. Viral RNA was then extracted with a commercial viral RNA extraction kit and quantified with qRT-PCR as described in the prior section.

2.6. ISC-qRT-PCR. ISC-qRT-PCR was performed as described previously (Wang et al., 2014; Wang et al., 2013). Porcine gastric mucin (PGM) or polyclonal antibodies against TV were used to capture encapsidated viral genome. PGM was purchased from Sigma (St. Louis, MI). Polyclonal antibodies against TV was kindly provided by Dr. Xi Jiang (Cincinnati Children Hospital, Cincinnati, OH). One hundred microliters of PGM (100 mg/mL) or 100 μ L of polyclonal antibody dilution (1:6,000 in 0.5M carbonate-bicarbonate buffer, pH 9.6) was added to each well of a Nunc Top-Yield module strip (VWR, Brisbane, CA) to coat its binding surfaces, and incubated at 4 °C for overnight. The antibody/PGM dilution was then removed and the binding surface was blocked with 10% dry milk at 37 °C for 1 hour. The wells are emptied of the blocking solution and rinsed with PBS immediately prior to use. One-hundred-microliters of TV stock was added to each antibody/PGM-coated well and incubated at 37 °C for 1 h. The wells are then emptied of the TV stock, rinsed three times with PBS, and dried in a fume hood. From a prior-prepared qRT-PCR master mix (described previously), 25 μ L is added to each well, sealed with polyolefin film, and quantified by qRT-PCR as described previously.

2.7. Conversion of genomic copy numbers (GCs) from Ct value. A p289/p290-based TV amplicon in a TOPO vector was used to generate a standard curve (Tian et al., 2013). The

plasmid DNA was serially-diluted to a theoretical range of 10^7 to 10^{-1} copies. A linear trendline was generated ($R^2 = 0.992$).

2.8. Data analysis and statistics. Each plating experiment was repeated three times as independent replicates. Each experiment contained at least three replicates. One-way ANOVA was employed for data analysis. SigmaPlot (Systat Software Inc., San Jose, CA) was used for simple or multiple regression analysis. Multiple regression analysis was used to determine a linear relationship between two assays. An R value greater than 0.7 indicates a strong positive linear relationship (<http://www.dummies.com/how-to/content/how-to-interpret-a-correlation-coefficient-r.html>).

3. Results

3.1. Data summary. Ten batches of viral stocks ranging from 3.41×10^5 to 6.67×10^6 PFUs were used for side by side comparison with PFU as a reference (Table 1). The ten batches of viral stocks were arranged and named in ascending order, with the bottom of the range (3.41×10^5 PFUs) defined as “batch #1”, and the top of the range (6.67×10^6 PFUs) defined as “batch #10”. As measured by TCID₅₀, viral titer ranged from 2.53×10^6 to 3.14×10^7 . On average, one PFU was equivalent to 6.7 ± 2.3 TCID₅₀ units. qRT-PCR quantitation of viral stocks produced a range between 7.89×10^6 to 1.18×10^7 GC units. qRT-PCR quantitation of viral stocks treated with RNase produced a range between 2.41×10^6 to 3.89×10^6 GC units. On average, one PFU was equivalent to 9.8 ± 10.9 GCs of qRT-PCR-quantitated viral stocks, and 2.8 ± 3.0 GCs of qRT-PCR-quantitated RNase-treated viral stocks. GC units ranged from 6.56×10^3 to 8.44×10^4 as measured by PGM-ISC-qRT-PCR, and from 1.82×10^4 to 8.46×10^5 as measured by Ab-ISC-qRT-PCR. On average, one PFU was equal to 0.07 ± 0.07 GC units for PGM-ISC-qRT-PCR, and 0.52 ± 0.39 GC units for Ab-ISC-qRT-PCR.

3.2. Plaque assay. Plaque assays were quantified 3 days after viral inoculation, as viral plaques grew to the point of merging after 4 days, which made counting problematic. The virus titers were $(3.41 \pm 1.26) \times 10^5$, $(3.83 \pm 0.65) \times 10^5$, $(6.21 \pm 2.22) \times 10^5$, $(1.87 \pm 1.38) \times 10^6$, $(2.30 \pm 1.50) \times 10^5$, $(2.63 \pm 0.42) \times 10^6$, $(2.66 \pm 0.39) \times 10^6$, $(2.66 \pm 1.53) \times 10^6$, $(4.32 \pm 0.71) \times 10^6$, and $(6.67 \pm 0.71) \times 10^6$ from batch 1 to batch 10, respectively. The R value of linear regression for plaque assay was 0.922.

3.3. Kinetics of TCID₅₀ assay. TCID₅₀ was performed with both M199 media with 0% FBS and M199 with 5% FBS. Plates were read starting from the 3rd day of incubation, through the 7th

day. Using the 7th day as the reference, TCID₅₀ titers using M199 media were 19.9%, 50.6%, 77.0%, 96.8% and 100% from incubation days 3 through 7, respectively. Again using the 7th day as the reference, TCID₅₀ titers using M199 with 5% FBS were 21.0%, 35.3%, 59.2%, and 84.0%, and 100% from incubation days 3 through 7, respectively. Higher titers overall were observed when TCID₅₀ was conducted using M199 media than M199 with 5% FBS. For TCID₅₀ conducted using M199, viral titers at incubation day 6 were not significant different from that of incubation day 7 ($p=0.77$). Therefore, we used data from this incubation day 6 using M199 media as representative of the TCID₅₀ assay for comparison against and analysis with other assays. The R value of linear regression for TCID₅₀ assay was 0.76.

3.4. Comparison of PFU and TCID₅₀. A strong positive linear relationship was found between results from the TCID₅₀ assay and the plaque assay (Fig. 1). The ratios between TCID₅₀ units vs PFUs were 7.43, 6.78, 5.06, 8.46, 11.42, 7.84, 4.18, 3.83, 7.26, and 4.60 for batches #1 through #10 respectively. The average TCID₅₀ unit to PFU ratio was 6.7 ± 2.3 . The R value for the multiple regression analysis (PFU vs TCID₅₀) was 0.85.

3.5. Comparison of PFU and RNase exposure assay. The proportion of RNase-protected encapsidated viral RNA that exists within the total viral RNA for each of the viral batches #1 through #10 were 32%, 22.5%, 37.1%, 33%, 26.5%, 30.5%, 30.5%, 28.9%, and 28.8%, respectively. The average proportion of encapsidated viral RNA from total viral RNA was $30.1 \pm 3.9\%$. There was no strong positive linear relationship between PFUs and viral RNA GCs with RNase (Fig. 2) or without RNase treatment (data not shown). Unlike PFUs, the ranges of GCs were very narrow. Without RNase treatment, the GC extremes were 1.18×10^7 (batch #2), and 7.89×10^6 (batch #7), which is only a factor of 1.5. With RNase treatment, the GC extremes were 3.89×10^6 (batch #3) and 2.41×10^6 (batch #7), which is only a factor of 1.58. There was

no statistical difference in GCs among 10 batches of viral stocks without RNase treatment ($p=0.574$) and among 10 batches of viral stocks with RNase treatment ($p=0.233$). There was a poor linear relationship between PFU and GCs where the R values for the multiple regression analysis were 0.09 (PFU vs. GCs without RNase treatment), and 0.22 (PFU vs. GCs with RNase treatment).

3.6. Comparison PFU and ISC-qRT-PCR assays. Overall, there was a poor positive linear relationship between the results from the plaque assay and the results from the ISC-qRT-PCR assays coated with either antibody (Fig. 3A) or PGM (Fig. 3B). The R values for the multiple regression analysis were 0.64 (PFU vs. GCs by Ab-ISC-qRT-PCR), and 0.56 (PFU vs. GCs by PGM-ISC-qRT-PCR). The GCs fluctuated widely in viral stocks measured by Ab-ISC-qRT-PCR. There was no statistical difference in GCs among 10 batches tested by Ab-ISC-qRT-PCR ($p=0.454$). A higher PFU was not reflected by a higher GC as measured by Ab-ISC-qRT-PCR assays. With Ab-ISC-qRT-PCR assays, batch #2 measured the highest in GC (8.46×10^5) and batch #10 the lowest (1.82×10^4). However, batch #10 measured at a higher PFU (6.67×10^6) than batch #2 (3.83×10^5). Similarly, the GCs also fluctuated widely in viral stocks measured by PGM-ISC-qRT-PCR. There was no statistical difference in GCs among 10 batches tested by PGM-ISC-qRT-PCR ($p=0.657$). A higher PFU was not reflected by a higher GC as measured by PGM-ISC-qRT-PCR assays. With PGM-ISC-qRT-PCR assays, batch #1 measured the highest in GC (8.44×10^4), and batch #5 the lowest (6.56×10^3). However, batch #5 measured at a higher PFU (2.30×10^6) than batch #1 (3.41×10^5).

4. Discussion:

Both plaque assay and TCID₅₀ assay are widely-used for the determination of infectivity of culturable viruses. Both assays have been used to quantitate TV in various laboratories. Plaques assays were generally counted between 2 to 4 days after inoculation (Fan et al., 2013; Hirneisen and Kniel, 2013; Wang et al., 2014; Dicaprio et al., 2012; Farkas et al., 2010; Tian et al., 2013), while TCID₅₀ assays were generally read for CPEs 4 to 6 days after inoculation (Tian et al., 2013; Wang et al., 2014; Xu et al., 2015). Our kinetics study showed that our best results for plaque assays were obtained at 3 days after inoculation, while that for TCID₅₀ assays were obtained at 6 days after inoculation. In general, we found plaques to be still too small 2 days after inoculation, and often overgrown to fuse with nearby plaques 4 days after inoculation (data not shown). Quantitation of viral titer via TCID₅₀ using M199 media always produced a higher measured titer than the same quantitation via TCID₅₀ using M199 with 5% FBS. We observed a significant increase in measured titer on the 6th day after inoculation relative to the 5th day after inoculation ($p < 0.05$), and no difference between 6th day and 7th day after inoculation ($p > 0.05$). In fact, TCID₅₀ at 6th day after inoculation was 26% higher than that of 5 days pi. Therefore, the actual virus titer might be higher than reported in literature if the TCID₅₀ was terminated at 5 days pi.

There is a strong positive relationship between the two cell-based assays, where the R value was 0.85. Under the conditions tested, the TCID₅₀ assay consistently yielded a higher titer than the plaque assay. On average for the same sample, the TCID₅₀ assay will produce a viral count about 6.7 times higher than the viral count produced by the plaque assay. The 3 days necessary for the plaque assay is faster than the 6 days required by the TCID₅₀ assay, but reduced sensitivity is the tradeoff. In general, TCID₅₀ assay is more sensitive than plaque assay. The

establishment of a mathematical relationship between these assays will now allow the conversion of data between the two units and enable comparison of historical data. However, experimental variations in different laboratories should be considered when the conversion is made.

In this study, we tested three different approaches aimed at improving the relevancy of molecular-technique-based quantitation of TV by screening for encapsidated viral genomes. The RNase exposure assay utilized RNase to remove any free RNA from the sample prior to quantitation. The PGM-ISC-qRT-PCR assay utilized PGM to segregate encapsidated viral genomes possessing functional receptors from the sample prior to quantitation. The Ab-ISC-qRT-PCR assay utilized antibodies to segregate encapsidated viral genomes (regardless of receptors) from the sample prior to quantitation. The correlation between these assays to PFU were very poor. By multiple regression analysis, the R values were less than 0.17 when plaque assay was compared with RNase protection assay and less than 0.64 when plaque assay was compared with ISC-qRT-PCR assays. The RNase treatment could reduce GC units by 70%, indicating that encapsidated viral genome might only account for 30% of the GC units. There was no statistical difference in GCs among the 10 batches tested by qRT-PCR with or among 10 batches without RNase treatment ($P=0.23$ and 0.57 , respectively). Likewise, there was no statistical difference in GC units measured by PGM-ISC-qRT-PCR assays ($P=0.66$) or An-ISC-RT-qPCR assays ($P=0.45$) among 10 batches of viral stocks. Overall, we failed to demonstrate strong positive linear relationship between the established cell-based assays and the encapsidated-genome screening PCR-based assays.

Several reasons may explain the difficulty in establishing a positive linear relationship between the cell-based and PCR-based assays. Despite a fairly narrow range of viral titers (as measured by PFUs), we experienced exceedingly large variations in assay measurements. The

10 batches of viral stocks used in this study ranged from 3.4×10^5 to 6.7×10^6 PFUs, with a high to low ratio of 19.7. Each viral stock were quantitated by 6 different methods and compared side by side. In the case of a single viral stock, relationships between the output of the assays could be easily calculated (Table 1). But when these assay output relationships between the 10 viral stocks were compared, we found huge variations. This problem was particularly important for the PCR-based assays. Smither et al reported results comparing the plaque and TCID₅₀ assays using filoviruses (Smither et al., 2013). In his study, a single viral stock was tested 10 times with triplicates. PFU titers ranged from 1×10^5 to 1.2×10^6 , spanning a 1.8-log difference over the range. TCID₅₀ titers ranged from 3.97×10^5 to 2.5×10^7 , spanning a 1.2-log difference over the range. We did not observe that magnitude of variation within our plaque and TCID₅₀ assays, but did in our ISC-qRT-PCR and RNase exposure assays. Interestingly, within a narrow range (batches #5 through #9, 2.3×10^6 to 4.2×10^6 PFU), there was a proportional relationship between PFU and GC units as measured by PGM-ISC-qRT-PCR assay, but not by Ab-ISC-qRT-PCR assay. Within this narrow range and measured by PGM-ISC-qRT-PCR assay, 1 PFU was equivalent to 0.05 ± 0.01 GC units. Outside of this range, there were huge variations. In batches with low PFUs, the equivalency ratio with GC units were high, and in batches with high PFUs, the equivalency ratio with GC units were low. The correlation between cell-based and PGM-ISC-qRT-PCR assay needs to be re-evaluated if these methods can be improved by reducing assay variation.

There were also limitations with the PCR-based assays. The PCR-based assays tested in this paper mainly focused on the detection of encapsidated viral genome. These relevance of these assays were based upon the theory that integritous viral particles were required for infection. When a virus loses capsid integrity, its genome becomes sensitive to RNase treatment.

Similarly, when a virus loses its ability to bind to its receptor, it cannot enter host cells to replicate. These assays have been successfully utilized to estimate inactivation status of the virus caused by capsid damage (Dancho et al. 2012; Nowak et al., 2011; Wang et al., 2014). In these published studies, a single batch of viral stock was tested before or after treatment. Selection of a single batch to test could effectively reduce variations and make comparison relatively easier. Furthermore, all these assays were formed in extern conditions to completely destroy viral capsid while greater than 3-4 logs difference between inactivated and non-inactivated viral samples which made comparison easier. However, these assays might not be entirely relevant for determining viral infectivity. Multiple factors might contribute to viral infectivity, with receptor binding or RNase resistance comprising only one factor among several. It remains unknown if HBGAs are exclusively required for viral infection. Recently, sialic acid has been identified as receptors/co-receptors for infection and replication of TV in the gastrointestinal tract of rhesus macaques (Tan et al, 2015). Write et al. reported that recombinant Norwalk viral particles could bind non-HBGA-expressing cells (White et al, 1996). It is possible that TV might use receptors other than HBGA to enter cells. Wei et al. reported that PCR-based TV RNA transcribed from full-length TV cDNA were infectious upon transfection into permissive LLC-MK2 cells (Wei et al., 2008). It is also possible that viral RNA itself could be infectious.

In summary, two cell-based assays were consistent with each other, with the TCID₅₀ assay was more sensitive than the plaque assay. There was a strong positive linear relationship between PFU and TCID₅₀ assays. In contrast, the PCR-based assays were not proportional consistent with the cell-based assays. There was no strong positive linear relationship between the cell-based assays and PCR-based assays.

Acknowledgements: This work was supported by USDA Agricultural Research Service CRIS project 5325-42000-046-06R . USDA is an equal opportunity provider and employer.

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Table and Figure Captions.

Table 1. Data summary. PFU was used for plaque assay. TCID₅₀ unit was used for TCID₅₀ assay. GCs were used for qRT-PCR assays with RNase treatment (RNase+) or without RNase treatment (RNase-), and ISC-qRT-PCR assay coated with PGM or antibody.

Figure 1. Comparison of plaque assay and TCID₅₀ assay.

Figure 2. Comparison of plaque assay and RNase exposure assay.

Figure 3. Comparison of plaque assay and PGM-ISC-qRT-PCR assay (A) or Ab-ICS-qRT-PCR assay (B).

Table 1.

Batch	plaque assay	TCID50 (0%)		Rnase +		Rnase -		ISC-qRT-PCR			
			Rati o to pfu		Rati o to pfu		Rati o to pfu	PGM	Rati o to PFU	Anti body	Rati o to Pfu
1	3.41E+05	2.53E+06	7.43	2.86E+06	8.39	8.94E+06	26.23	8.44E+04	0.23	4.54E+05	0.53
2	3.83E+05	2.60E+06	6.78	2.65E+06	6.92	1.18E+07	30.83	4.08E+04	0.10	8.46E+05	1.37
3	6.21E+05	3.14E+06	5.06	3.89E+06	6.26	1.05E+07	16.87	4.91E+04	0.12	5.54E+05	0.91
4	1.87E+06	1.58E+07	8.46	2.96E+06	1.58	9.52E+06	5.09	5.07E+04	0.03	7.03E+05	0.12
5	2.30E+06	2.63E+07	11.42	3.80E+06	1.65	1.15E+07	5.01	6.56E+03	0.05	3.07E+05	0.50
6	2.63E+06	2.06E+07	7.84	2.46E+06	0.93	9.28E+06	3.53	1.31E+04	0.05	3.16E+05	0.60
7	2.66E+06	1.11E+07	4.18	2.41E+06	0.90	7.89E+06	2.97	3.60E+04	0.06	7.56E+04	0.49
8	2.66E+06	1.02E+07	3.82	2.78E+06	1.04	9.11E+06	3.42	7.50E+04	0.04	7.91E+05	0.24
9	4.32E+06	3.14E+07	7.26	2.72E+06	0.63	9.43E+06	2.18	1.57E+04	0.02	3.54E+05	0.31
10	6.67E+06	3.07E+07	4.60	2.66E+06	0.40	9.27E+06	1.39	1.54E+04	0.00	1.82E+04	0.10
Highest	6.67E+06	3.14E+07		3.89E+06		1.18E+07		8.44E+04		8.46E+05	
Lowest	3.41E+05	2.53E+06		2.41E+06		7.89E+06		6.56E+03		1.82E+04	
Mean	3.50E+06	1.55E+07		2.92E+06		9.72E+06		3.87E+04		4.42E+05	
Rati o (mean)	1		6.69		2.87		9.75		0.07		0.52
SD			2.34		3.05		10.87		0.07		0.39

Fig. 1

Fig. 2

Fig. 3A

Fig. 3B