Is PMAxx an appropriate culture-independent viability assay for human Norovirus?

Samantha Q. Wales and Michael Kulka
CFSAN/OARSA/DMB 8301 Muirkirk Rd Laurel MD 20708

Abstract

Plain Language Synopsis: PMAxx is an intercalating dye that has been used as a culture-independent method for determining the viability of several species of bacteria. We wanted to determine if it holds promise for determining the infectivity of human norovirus using human intestinal enteroids (HIEs) to confirm virus viability.
Background: **Because the culture methods currently in use for NoV suffer from limitations including strain preference and the requirement of high titer NoV for successful replication, it is important to have the ability to confirm the infectivity of the virus with a culture-independent method**. PMAxx is an intercalating dye that is widely used to discriminate between viable and non-viable bacterial species. More recently it has been used by several groups to determine viability of human NoV.
Purpose: We wanted to determine if PMAxx treatment of heat-inactivated NoV could accurately predict the infectivity of human NoV using human intestinal enteroids to test virus replication post heat-treatment.
Methodology: GII.4[P16] or GII.6[P7] were heat inactivated at 60 or 95°C for ten minutes or kept on ice as a control (non-treated). Heat-treated and control viruses were then divided for either PMAxx treatment or for virus infection studies. RNA generated from some of the studies were subjected to RT-long PCR and gel electrophoresis to determine if the viral genome was intact after PMAxx treatment. PMAxx was also tested on a GI.3 NoV at 60, 72 and 88°C, though this strain does not grow in J2 and confirmation of infectivity could not be determined.
Results: We showed that 60°C was sufficient to inhibit GII virus replication, but RT-qPCR of PMAxx-treated samples showed no reduction in quantity, falsely indicating that the virus is still viable. RT-long PCR demonstrated full-length GII.4 genome in both untreated and 60°C-inactivated virus. GI.3 virus only showed a reduction in RT-qPCR at 88°C.
Conclusion: We wanted to confirm PMAxx determination of viability in J2 human intestinal enteroids using two GII strains. Our results indicate that PMAxx is not an appropriate culture-independent assay for determining viability of human norovirus. The data indicate that inactivation at 60°C likely causes damage to the capsid that inhibits replication but does not allow for dye penetration.

Introduction

Infection with human Norovirus (NoV) can occur with less than 100 virus particles. The amount of NoV that is generally recovered from naturally contaminated foods are typically in this range or even lower, and are detected by RT-qPCR assays, which are extremely sensitive but do not give any information on viability of the virus. The culture system for NoV suffers from limitations in that not all NoV genotypes can grow, and there is a requirement for high titer virus for successful infection. Thus, there is a need for an assay that is culture-independent but gives information on virus infectivity. PMAxx is a dye that can only enter compromised/damaged virions, and will covalently bind to viral genomes and interfere with RT-qPCR once bound, preventing amplification of viral genomes. It has been successfully used to determine bacterial viability. We wanted to see if it would be suitable for determination of NoV infectivity, using the human intestinal enteroids culture system to confirm virus viability post heat-treatment.

Materials and Methods

Viruses and cells: GII.4[P16], GII.6[P7], or GI.3[P3] virus strains were all single case volunteer samples, approved for use by FDA IRB 17-048. All stools were made as 10% suspensions in PBS, clarified by centrifugation at 13,000 x g, followed by filtration in 0.2 µM filters. Virus suspensions were subjected to indicated heat treatments for 10 minutes, then aliquoted for either PMAxx treatment or cell infection. J2 HIEs were maintained and differentiated as described in Ettayebi et al, 2021. mSphere6:e01136-20. For virus infections, 100 µL of each virus at the indicated treatment was added per well and adsorbed to cells for 1.5h. After the adsorption period, virus was removed and cell monolayers were washed 3 times prior to addition of differentiation media containing GCDCA. At this time the "O hpi" time point was collected and frozen until RNA extraction could be performed. At 3 dpi the additional wells were frozen until RNA extraction and subsequent RT-qPCR analysis.
PMAxx assay: Virus suspensions or GII.4[P16] RNA transcripts were subjected to indicated heat treatments, then mixed with Triton-X 100, PMAxx (Biotium), or Enhancement buffer as described in figure legends, shaken in the dark for 15 minutes at 450 rpm on a shaking platform, and then subjected to photoactivation with the PMA-Lite™ LED device for 15 min. Samples were then extracted for RNA and frozen until RT-qPCR could be performed.
RT-qPCR: Reverse transcription quantitative PCR was performed as described in Ford-Siltz et al 2022 JID 2022;225:1205–14.
Reverse transcription full length PCR (RT-FL PCR): RT-FL PCR was performed using an oligodT primer for reverse transcription, followed by PCR using a primer targeted at the 5' end as well as oligo dT. The samples were then run on agarose gel electrophoresis to visualize the amplicons generated.

Results and Discussion

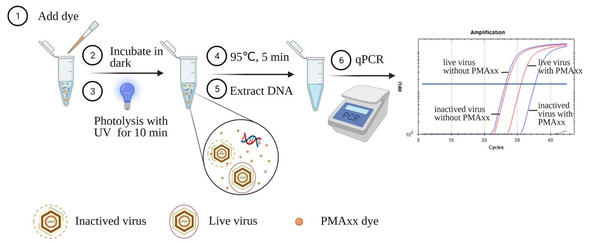

Figure 1. Schematic of PMAxx assay from: Front. Microbiol., 05 December 2022 Sec. Infectious Agents and Disease, Volume 13 - 2022. Inactivated viruses have damaged capsids that can allow access to the viral genome. The dye is added to the sample, incubated with shaking in the dark, and activated with strong light to induce covalent binding of the dye to the nucleic acids that it has access to. RNA is extracted and qPCR performed on the samples. Because the genome of norovirus is fairly stable, even heat inactivation, depending on the temperature, may not reduce the PCR signal (shown here as the amplification curve of inactivated virus without PMAxx). Inactivated virus with PMAxx has the highest CT value and is shifted farthest to the right, indicating that the dye had access to the genome and reduced PCR amplification

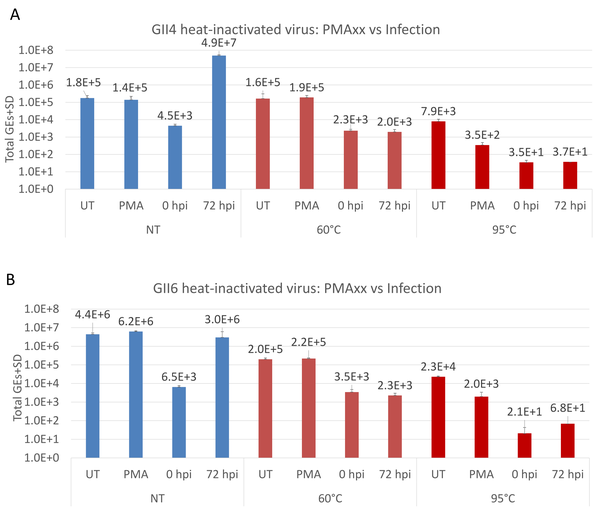

Figure 2. Viruses (A, GII.4[P16] and B, GII.6[P7]) were either not treated (NT), or heated to 60 or 95°C, then aliquoted as described in Materials and Methods, and combined with 200 µM PMAxx (PMA) or not (UT) and subjected to photoactivation prior to RNA extraction, or added to 5 day differentiated J2 monolayers (0 hpi or 72 hpi). Only NT viruses showed replication (as indicated by 3-4 log increases at 72 hpi vs 0 hpi), while 60°C was sufficient to render the virus inactivated. However, only treatment at 95°C showed a difference between PMAxx- and untreated virus, and even that was only 1 log difference.

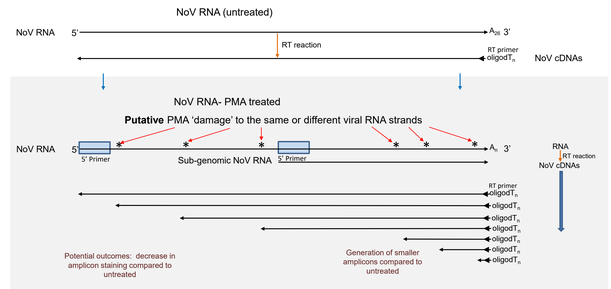

Figure 3. Schematic describing the RT-FL PCR and predicted outcomes. We reasoned that that if we subjected PMAxx-treated samples to this RT and PCR, if the PMAxx was binding to other areas of the genome aside from just where the PCR primers and probes are, we would see either truncated amplicons of varying sizes, or perhaps a reduction in intensity of amplicon signal.

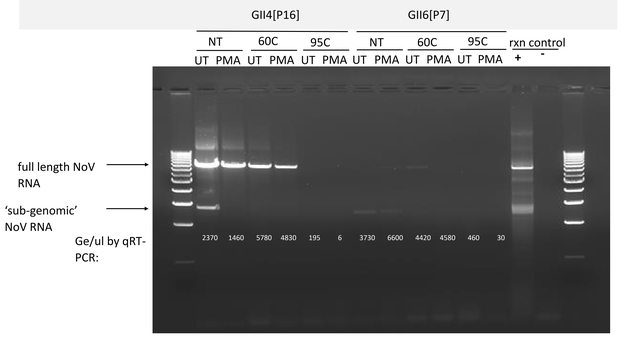

Figure 4. The results of the RT-FL PCR performed on RNA from PMAxx- or UT heat-inactivated or control viruses. The GII.6[P7] virus does not amplify well in this assay which is why we do not see bands in any of the samples. The GII.4[P16] shows a strong band in the non-heat treated samples (NT), though it is interesting to note that the sub-genomic RNA disappears with PMAxx treatment. However, there was no generation of smaller amplicons, and the reduction in intensity between UT and PMAxx-treated samples subjected to 60°C treatment was minimal, while 95°C treatment completely abrogated amplification by this method.

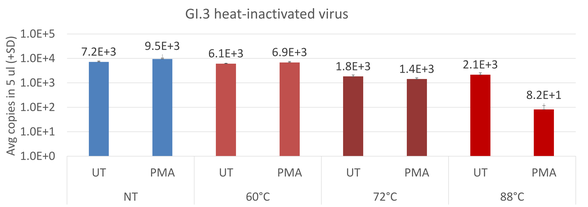

Figure 5. We repeated the PMAxx assay with a GI.3[P3] virus, as the literature indicated that GI viruses may be more amenable to PMAxx treatment as compared to GII.4 viruses, and we added 0.1% Triton-X to the assay. Because we did not have a GI virus that grows in the HIEs, infectivity could not be measured, which is why we added the 72°C treatment, as this temperature should be sufficient to inactivate GI, if 60°C is not. Again we saw little difference in PMAxx-treated samples until very high temperatures (88°C) were used to inactivate the virus.

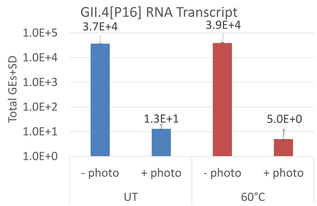

Figure 6. To ensure that the PMAxx assay is working correctly, I used RNA transcript and subjected it to 100 µM PMAxx with the addition of 0.5% Triton-X and Enhancement Buffer (Biotium). The control in this experiment omitted the photoactivation step rather than the omission of PMAxx. Photoactivation resulted in 3-4 log reduction in genome equivalents, showing that the dye is capable of interfering with PCR amplification.

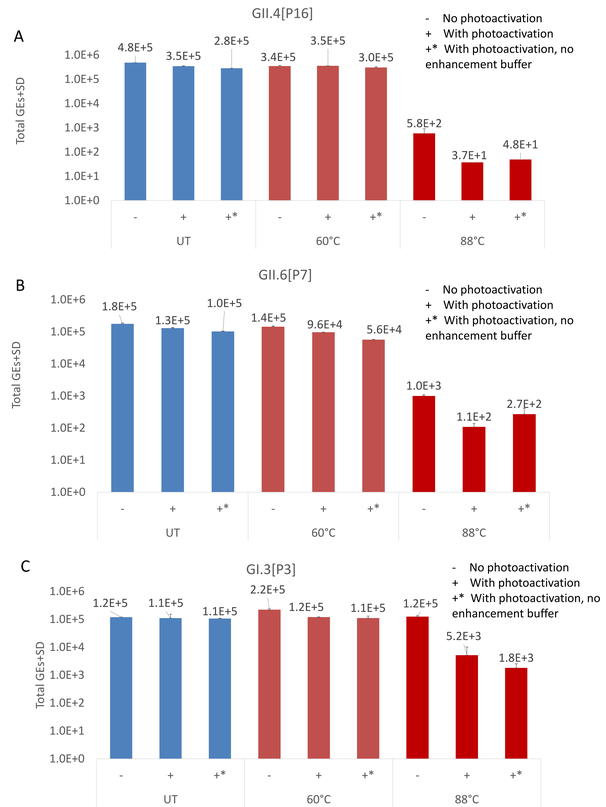

Figure 7. Use of the Enhancement Buffer, 0.5% Triton-X, and 100 µM PMAxx on intact viruses (A, GII.4[P16]; B, GII.6[P7], and C, GI.3[P3]) still does not accurately reflect infectivity.

Conclusions

60°C is sufficient to inactivate both GII.4[P16] and GII.6[P7] viruses, but PMAxx-treatment at these temperatures is relatively unchanged. PMAxx treatment of RNA transcript alone does yield 3-log difference with and without photoactivation, so the assay is operational. Enhancer buffer and Triton-X addition do little to improve the assay for GII.4, GII.6, and GI.3 NoV. The data indicate that a viability assay that can more accurately detect subtle capsid changes might predict virus viability with more accuracy