Aquavalens Project

"Protecting the health of Europeans by improving methods for the detection of pathogens in drinking water and water used in food preparation."

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Deliverable D2.3

Provide a correct estimation of infectious viruses through determination of genome copy numbers.

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D 2.3. Provide a correct estimation of infectious viruses through determination of genome copy numbers.

Background.

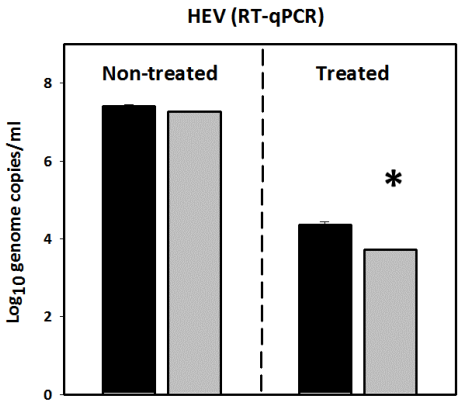
The advent of molecular methods enabled the development of diagnostic tools with exquisite sensitivity for detection of human pathogenic viruses that do now grow in cell cultures. In this way the significance of fastidious enteric viruses such as hepatitis A virus (HAV), hepatitis E virus (HEV), rotaviruses (RV) and, particularly, noroviruses (NoV) as water and foodborne agents could be ascertained (Lopman et al., 2004; Lopman et al., 2003; Mead et al., 1999).

Two ISO proposals (ISO/TS 15216-1 and ISO/TS 15216-2) for sensitive qualitative and quantitative determination of HAV and NoV in water and several food matrices have been recently published and could enable the formulation of regulatory standards for viruses in food and water. These methods are based on quantitative real time RT-qPCR, which is fast and extremely sensitive, but since detected viral genomes are not necessarily infectious, molecular detection assays need to be refined to better predict infectivity of contaminated samples.

Several approaches have been used in Aquavalens to estimate infectious virus levels through determination of genome copies. These approaches are based on the assessment of the integrity of the virion capsid trough treatments prior to the RT-qPCR assay such as RNase digestion, use of nucleic acids intercalating dyes or binding to gastric mucin.

RNase pretreatments

Ribonuclease (RNase) pretreatment was described for the successful differentiation between infectious and non-infectious virus (Nuanualsuwan and Cliver, 2002) although other studies failed to show correlation between infectious units and genome copies (gc) for murine norovirus which may be propagated in macrophage cell monolayers (Baert et al., 2008). Actually, data produced in WP2 indicates that, as will be also evidenced with other treatments prior to genome amplification, there is no universal behaviour of viruses regarding the usefulness of RNase ONE digestion (20 and 40 U at 37ºC) performed before RT-qPCR to discriminate between infectious and physical particles. While no successful data were produced with human NoV or HAV, RNase ONE digestion prior to RT-qPCR led to a 3.0 to 4.5-log reductions in gc of HEV after 15-min heat treatment at 99ºC, thus providing a better estimation of infectious units (Figure 1 and Table 1).



* No Cq; the tLOD is employed for the plot.

Figure 1. HEV genome copy reduction after after 15-min heat treatment at 99º (Black bar: without RNase pretreatment; Grey bar: with RNase pretreatment).

Table 1. Genome copy reduction of HEV heat-treated and non-heat-treated for 15 min at 99ºC.

	RT-qPCR (log_{10} genome copies/mL)		
	Non-treated	Treated	**Reduction**
HEV	7.40 ± 0.04	4.35 ± 0.09	**3.05**
HEV + RNAse	7.26 ± 0.00	≤3.72	**≥3.54**
Reduction	0.14	≥0.63	

The main difficulty in ascertaining the validity of RNase pretreatment is that after heat treatment, with RNase, the gc numbers go below the limit of detection which makes impossible to accurately quantify the actual difference in gc due to the enzyme pretreatment.

Use of propydium monoazide (PMA) to discriminate infectious virus particles in RT-qPCR assays.

In the literature, PMA-treatment prior to RT-qPCR has been described for non-enveloped RNA viruses such as rotavirus, murine norovirus, poliovirus type1, echovirus 7, coxsackievirus B5, Snow Mountain virus and Norwalk virus (the last two both being prototypes of human norovirus) as a possible way to distinguish between intact and inactivated viruses after heat treatment (Coudray-Meunier et al., 2013; Parshionikar et al., 2010; Sánchez et al., 2012). Due to the increased capsid permeability of thermally inactivated viruses, PMA diffuses into capsid-damaged viruses where it intercalates with the nucleic acids. Upon light activation, PMA covalently binds to the nucleic acids and inhibits subsequent RT-qPCR amplification (Hixon and Yielding, 1975).

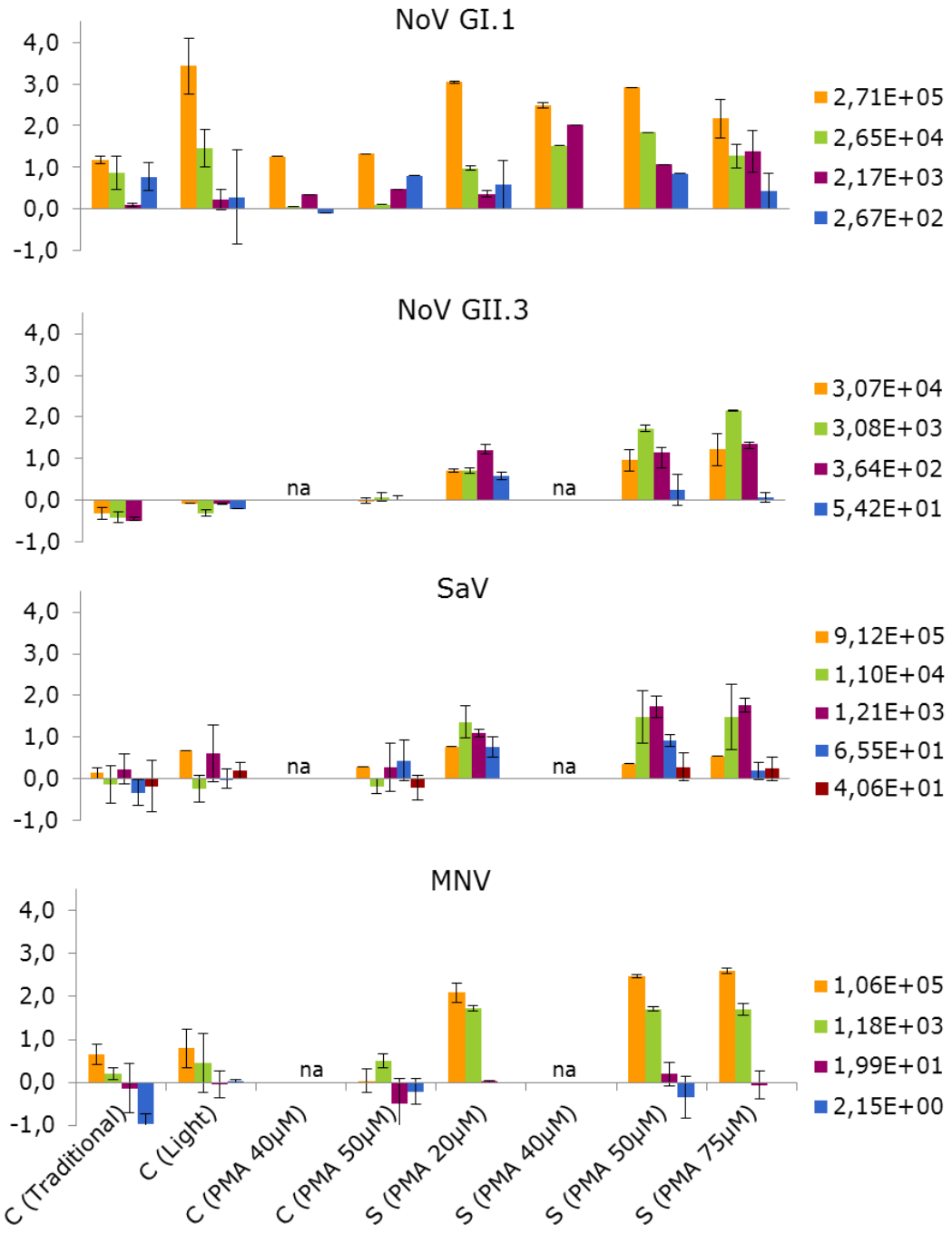


Figure 2. Reduction of viral genomes after heat treatment (80°C for 10 min) using various pretreatments to evaluate the efficacy of PMA-RT-qPCR to distinguish between intact and thermally inactivated virions. X-axis: Pretreatments applied for controls (C) and samples (S). Y-axis: Log Reductions in genome copies for NoVs or RT-qPCR units for SaV and MNV. Barcodes: Amount of virus in samples (genome copies for NoVs or RT-qPCR units for SaV and MNV) before thermal inactivation. na: not analysed.

In Aquavalens, PMA has been employed at different concentrations (20 µM, 50 µM, 75 µM, 100 µM, 150 µM and 200 µM) to differentiate infectious and non-infectious material detected by RT-qPCR. As an example, figure 2 (above) shows the decrease in viral genomes after heat treatment using various pretreatments to evaluate the efficacy of PMA-RT-qPCR to distinguish between intact and thermally inactivated NoV GI.1 and GII.3, as well as SaV and murine norovirus (MNV). This latter virus enables the comparison between genome copies and infectious units since it may be assayed by infectivity (Wobus et al., 2004).

Since apparently structured nucleic acids seem to be necessary for the successful discrimination of infectious material through the use of PMA (Parshionikar et al., 2010), HAV, which has a highly structured 5' NCD targeted for amplification, was extensively used to ascertain the validity of the methodology. With this aim, a method involving the use of PMA before RT-qPCR amplification was optimized for the detection and quantification of HAV with intact cohesive undamaged capsids. Addition of Triton-X100 as a surfactant during PMA incubation was also evaluated. The predictive value of the optimized protocol was validated using samples contaminated with HAV and inactivated by two different treatments. On the one hand, free chlorine (FC), which mainly alters the viral capsid but which can also damage specific genomic regions was employed, and on the other hand heat treatment at temperatures over 70ºC, which mainly affects virus proteins was also used. Figure 3 depicts the employed methodology.

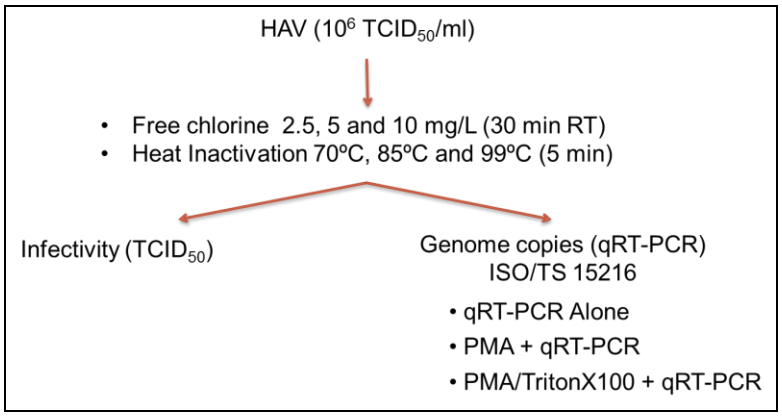


Figure 3. Methodology employed to ascertain the validity of PMA treatment applied prior to RT-qPCR for the determination of infectious HAV, after FC or heat treatments. The main objective was to verify the correlation between $TCID_{50}$ determination and gc quantification.

Results obtained with chlorinated samples.

Viral titers obtained by molecular and infectivity assays after inactivation are shown in Table 2. Low FC doses up to 2.5 mg/L did only affect the infectivity assay with a signal reduction of 1.34±0.45 log (Figure 4A). No effects were observed in any of the molecular tests used (RT-qPCR alone, PMA+RT-qPCR or PMA/Triton+RT-qPCR). With higher doses up to 5 and 10 mg/L of FC, infectivity was reduced over 4.5 logs, and in most cases viral titers decreased below the detection limit. Log reductions obtained by molecular tests were higher when PMA was included, but Triton-X100 did not result in a significant increase in log reduction.

These results indicate that low FC concentrations damage capsid surface proteins up to a point which is essential for infection. However, PMA treatment did not show any effect on the viral genome. At higher concentrations, chlorine damages the viral capsid allowing the penetration of PMA, resulting in additional signal reduction when using

RT-qPCR tests. Interestingly, since it has been documented that FC may specifically damage the 5' non-translated region (NTR) of the HAV genome (Li et al., 2002), the average log reduction obtained by RT-qPCR alone at 10 mg/L FC (1.90 ±0.87) did not differ as much from the reduction obtained by adding PMA pretreatment (2.71±1.03).

Table 2. Inactivation of HAV by free chlorine (FC) treatment as determined by molecular[a] and infectivity methods[b]. Titers are expressed as average ± standard deviation. Experiments were repeated 3-5 times.

	0 mg/L FC	2.5 mg/L FC	5 mg/L FC	10 mg/L FC
qRT-PCR Alone[a]	7.93 ± 0.52	8.04 ± 0.36	7.49 ± 0.75	6.32 ± 0.28
PMA+qRT-PCR[a]	8.03 ± 0.60	7.92 ± 0.44	6.11 ± 0.69	5.57 ± 0.69
PMA/Triton+qRT-PCR[a]	7.98 ± 0.53	7.76 ± 0.42	5.88 ± 0.71	5.86 ± 0.79
$TCID_{50}$[b]	6.16 ± 0.52	4.16 ± 0.26	2.30 ± 1.04	1.38 ± 0.08

[a] Titers are expressed as log genome copies/mL of sample
[b] Titers are expressed as log $TCID_{50}$/mL of sample

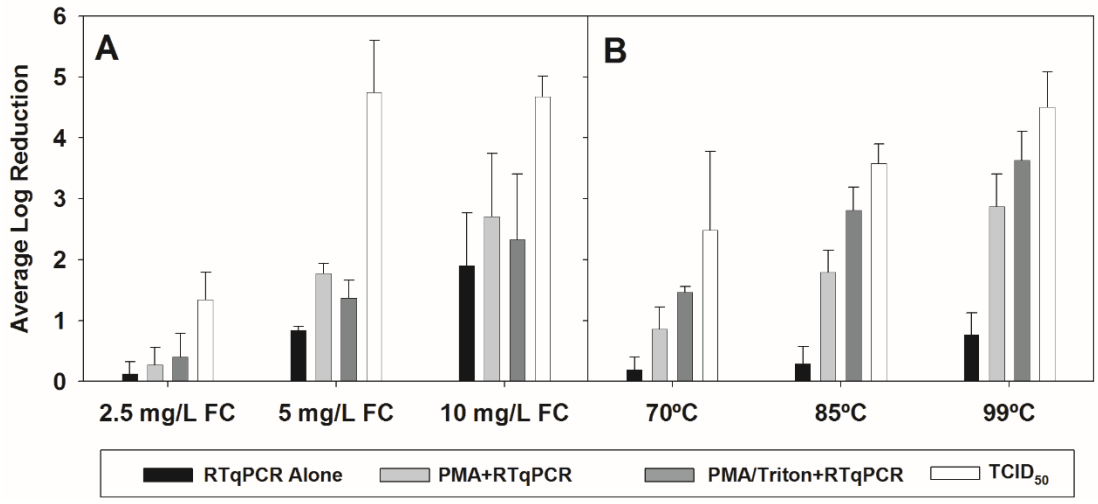


Figure 4. Average log reductions after HAV inactivation by free chlorine treatment (A) and heat treatment (B). Data are expressed as average ± standard deviation. Experiments were repeated 3-5 times.

Results obtained with heat-treated samples.

Viral titers obtained by molecular and infectivity assays after inactivation are shown in Table 3. After treatment at 70ºC, infectivity was reduced 2.48±1.30 logs (Figure 4B).

Treatments at 85ºC and 99ºC resulted in a loss of 3.58±0.32 and 4.50±0.58 logs of infectious viruses, respectively. Despite this high effect on infectivity, RT-qPCR alone only caused a reduction lower than 1 log in all cases, confirming that the main target for inactivation using high temperature is the viral capsid.

Table 3. Inactivation of HAV by heat treatment as determined by molecular[a] and infectivity methods[b]. Titers are expressed as average ± standard deviation. Experiments were repeated 3-5 times.

	RT	70ºC	85ºC	99ºC
qRT-PCR Alone[a]	8.83 ± 0.00	8.60 ± 0.26	8.58 ± 0.19	8.08 ± 0.46
PMA+qRT-PCR[a]	8.87 ± 0.24	7.92 ± 0.57	7.19 ± 0.45	5.99 ± 0.84
PMA/Triton+qRT-PCR[a]	8.58 ± 0.52	7.09 ± 0.59	6.06 ± 0.40	4.96 ± 0.42
$TCID_{50}$[b]	5.81 ± 0.53	3.07 ± 1.43	2.24 ± 0.79	≤1.43 ± 0.00

[a] Titers are expressed as log genome copies/ml of sample
[b] Titers are expressed as log $TCID_{50}$/ml of sample

Addition of PMA improved correlation between infectivity and molecular assays, and it was more effective in the presence of the surfactant agent (Fig. 4B). When assayed by PMA/Triton+RT-qPCR, log reductions obtained at 85ºC and 99ºC were 2.81±0.38 and 3.63±0.48, respectively, which only differ in less than 1 log with infectivity log reductions.

Correlation analysis

To assess the predictive values of the molecular test for each inactivation treatment, linear regression analyses were performed. Data were modeled using linear predictor functions and R^2 values were compared (Figure 5). Results indicate that viral inactivation by chlorine treatment could be better estimated by the PMA+RT-qPCR assay (Figure 5B), while inactivation by high temperature was better estimated by the

PMA/Triton+RT-qPCR assay (Figure 5F), confirming that addition of the surfactant was especially helpful to discriminate inactivated viruses damaged by heat.

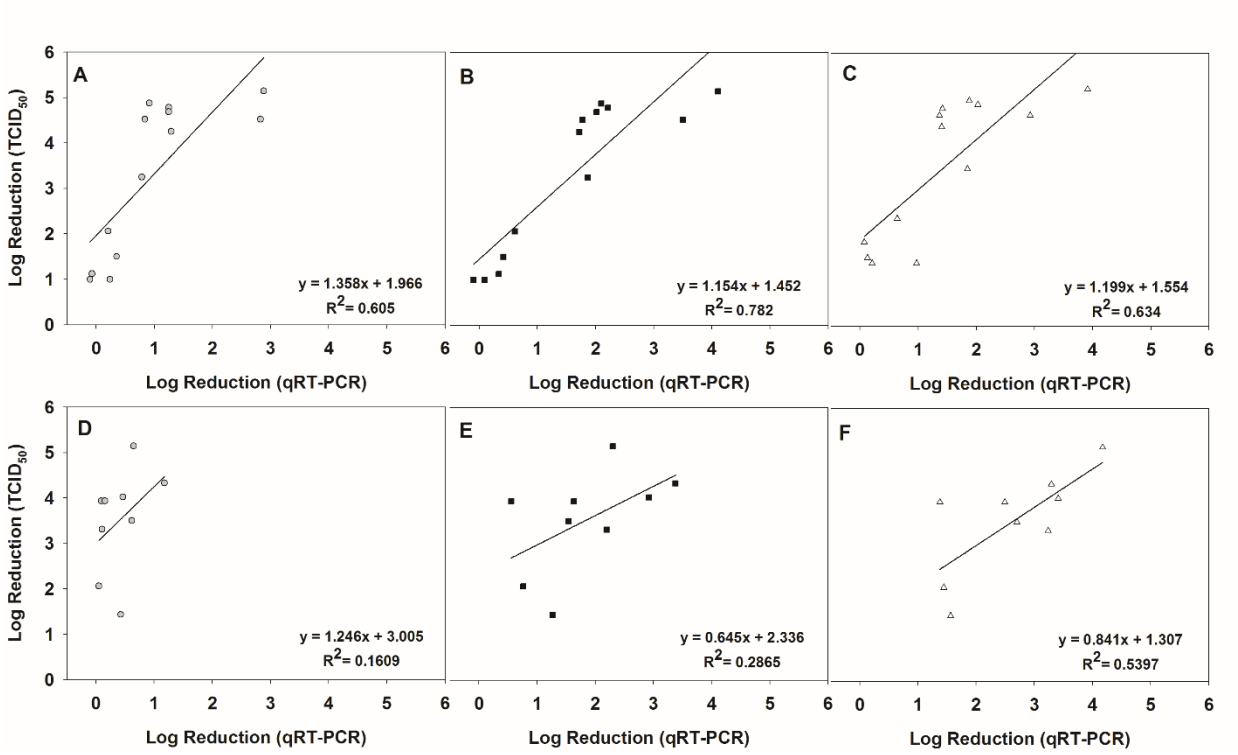


Figure 5. Correlation analysis between infectivity and RT-qPCR log reduction when using different methods for virus detection. (A-C) include results obtained by free chlorine inactivation and (D-F) include results obtained by heat treatment. (A) and (D) show correlation between infectivity and RT-qPCR alone, (B) and (E) show correlation between infectivity and PMA+RT-qPCR, and (C) and (F) show correlation between infectivity and PMA/Triton+RT-qPCR. Equations and R^2 values are shown for each plot.

These linear regressions may be used to estimate the level of infectious virus inactivation when using molecular methods, but it should be taken into account that correlation depends on the mechanism of inactivation. For example, a 4-log reduction in infectivity level corresponds to a 2.24 log gc reduction for chlorine-inactivated HAV and to a 3.20 log gc reduction for heat-inactivated HAV.

Effects of RNase and PMA treatments on heat-inactivated rotavirus.

RV strain Wa was propagated in MA104 cells and subsequently divided into two aliquots (3.3 x 10^3 $TCID_{50}$/ml). The first aliquot was used as control, while the other was treated with 3 U RNase (RNase from bovine pancreas, Sigma-Aldrich) for 15 minutes at 37 °C. This preliminary RNase treatment was performed in order to eliminate free RNA possibly present in the aliquot. Both aliquots were heat-inactivated at 80°C from 0 to 30 minutes. The infectivity decreased rapidly after 1 min incubation at 80°C, and after 5 min it became undetectable by the $TCID_{50}$ assay. In the RT-qPCR the viral titer remained relatively constant regardless of the inactivation time.

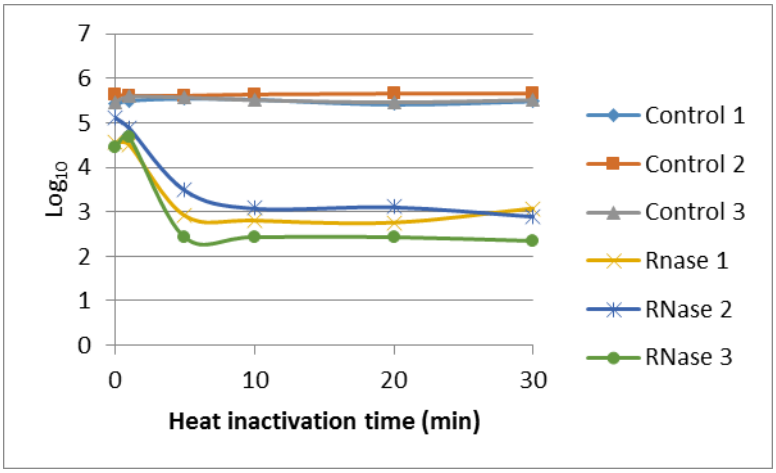


Figure 6. The effect of RNase (3 U) treatment on heat-inactivated RV strain Wa.

Several different time-temperature combinations for heat inactivation were tested with different concentrations of RNase or PMA. The sharpest log reduction (2.6 log_{10}) was observed when the samples of the first aliquot were heat-inactivated at 80°C for 5 minutes and subsequently treated with 3 U RNase (Figure 6). Statistically significant log reductions were also seen in the samples that were first treated with RNase, then

heat-inactivated at 80°C for 5 or 10 minutes and finally treated with 100 µM PMA prior to RT-qPCR (Figure 7).

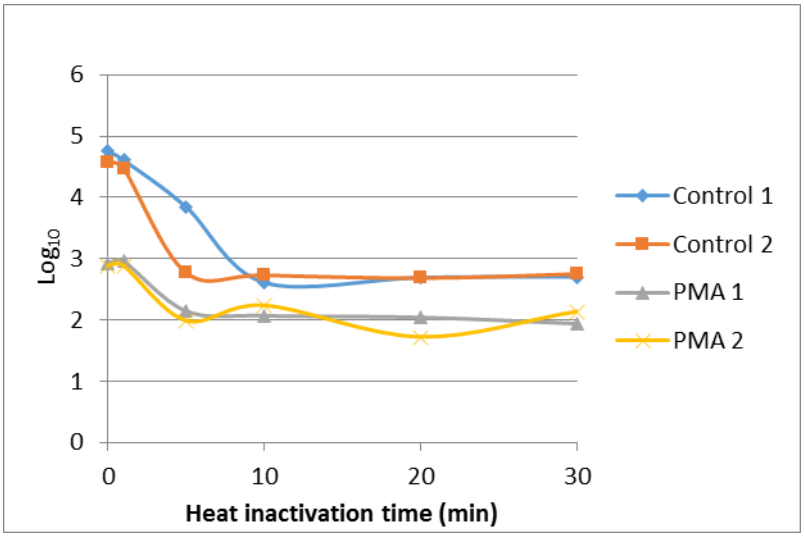


Figure 7. The effect of PMA (100 µM) on RNase-treated, heat-inactivated RV strain Wa.

Detection of viruses with intact surface proteins by use of mucin conjugated beads.

To facilitate the capture of infectious viruses, porcine gastric mucin (PGM) conjugated beads were tested for efficiency to catch viruses with intact surface proteins. NoVs bind to histo-blood group antigens (HBAg) expressed by epithelial cells in subsets of humans while MNV binds to sialic acids. PGM contain large, heterogeneous extracellular glycoproteins incl. 80% carbohydrates e.g. HBGAg and sialic acids. Mucin can thereby interact with intact viral capsids while damaged capsid proteins are deficient for binding. Previous studies have shown that PGM coupled to magnetic beads interact with 100% of tested strains of NoV GI and 85% of NoV GII (Tian et al., 2010). Moreover, PGM coated beads have been shown capable of binding on average to 68% of PCR detectable NoVs, while binding of heat treated NoVs were reduced to

6% (Dancho et al., 2012). In addition, the use of PGM-coated beads have resulted in an improved capture of NoV (increase of 2 log10) extracted from spiked food samples (Tian et al., 2008).

In a small scale study using 900 µL-volumes of 10-fold diluted NoV GI, NoV GII and MNV, PGM-coated beads were tested for the efficiency to capture viruses present in different levels without treatment and after being heat treated at 80 ^{O}C for 10 minutes. As shown in Table 4, the application of PGM-RT-qPCR on heat treated samples resulted in overall reductions in detectable viral genomes ranging from 58.51 to 3.97% for NoV GI and from 68.69 to 4.80% of NoV GII. For MNV, a reduction from 12.27 to 1.00% PCR detectable genomes could be observed in the heat-treated suspensions. The loss of intact viruses during PGM capture was determined by quantifying the remaining part of intact viruses present in the supernatant subjected to RNase treatment prior to nucleic acid extraction. This indicated an escape from PGM capture of a smaller fraction of viruses with intact capsids which might be due to destroyed surface proteins.

Table 4. Summary of overall percentages (mean ± SD) of PCR detectable viral genomes of intact capsids in viral suspensions captured by PGM-conjugated beads and lost in the RNAse treated supernatant.

	Viral capture by PGM (%)		Viral loss in RNase treated supernatant (%)		Total detected intact viral capsids (%)	
Virus	Untreated	Heat treated[a]	Untreated	Heat treated[a]	Untreated	Heat treated[a]
NoV GI	58.51 ± 16.22	3.97 ± 3.61	17.03 ± 8.81	2.41 ± 4.91	75.54	6.38
NoV GII	68.69 ± 45.43	4.80 ± 4.12	10.60 ± 5.83	0.64 ± 1.84	79.29	5.44
MNV	12.27 ± 43.31	1.00 ± 0.47	8.99 ± 8.23	0.98 ± 2.32	21.26	1.98

[a] Viral suspensions subjected to heat treatment at 80 ^{O}C for 10 minutes.

The data confirms the above mentioned study (Dancho et al., 2012) by showing that capturing of viruses using PGM coated beads, may indeed facilitate selection of viruses with intact surface proteins. However, this selection seems to be more efficient for NoV GI and GII which binds to cell ligands such as HBGA, than for MNV which applies sialic acids. The use of PGM may thus be a more reliable procedure compared to precipitation, which also results in detection of released genomes and destroyed (non-infectious) viral capsids. In addition, virus capture by PGM has been tested in WP6 in 45-mL suspensions of buffers (NaPP and GBEB) used for the elution of viruses from filtration of large scale volumes of water.

Conclusions.

Data from the different laboratories involved in these studies indicate that there is no universal pattern of behaviour for all viral strains assayed and for the inactivation treatments. The level of virus decay also seems to influence the correlation between infectivity and genome copies at least when employing PMA, i.e., the higher the virus infectivity decay the worse the estimation of infectivity through molecular assays.

It could be reasonable to point that PMA seems more promising with viruses with genomes bearing some kind of structure, such as picornaviruses, e.g., HAV, enterovirus, etc., or RV; while viruses with a very lineal genome, such as NoV and HEV, could perform worse with PMA, and RNase treatment could be a better option. RNase treatment seems to be a good option for HEV and for RV, alone or combined with PMA.

Addition of Triton-X100 during the PMA incubation step is helpful to discriminate viruses inactivated by high temperatures but does not improve correlation with infectivity for viruses inactivated by free chlorine.

Binding to PGM-coated beads seems to be an elegant way to discriminate intact Nov particles and seems more appropriate than PMA treatment for this purpose. However, the correction caused by this approach was below 70% (i.e., less than two logs) in heat-treated (80^{O}C for 10 min) samples, which clearly is far from reality.

The bottom line is that all these pretreatments may provide a better estimation of infectious units after molecular detection but still there is a several log difference between physical and infectious particles. Nevertheless, despite the molecular overestimation of virus infectivity, it is undeniable that in some scenarios, a more accurate measure of the risk associated with the occurrence of viruses in a given sample may certainly be inferred from the use of PMA, RNase or PGM binding (depending on the virus target) prior to genome detection.

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