Propidium Monoazide Reverse Transcriptase PCR and RT-qPCR for Detecting Infectious Enterovirus and Norovirus

Running Title: PMA RT-PCR to detect infectious enteric viruses

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Abstract

Presently there is no established cell line or small animal model that allows for the detection of infectious human norovirus. Current methods based on RT-PCR and RT-qPCR detect both infectious and non-infectious virus and thus the conclusions that may be drawn regarding the public health significance of positive findings are limited. In this study, PMA RT-PCR and RT-qPCR assays were evaluated for selective detection of infectious poliovirus, murine norovirus (MNV-1), and Norwalk virus. Viruses were inactivated using heat, chlorine, and ultraviolet light (UV). Infectious and non-infectious viruses were treated with PMA before RT-PCR and RT-qPCR. PMA RT-PCR was able to differentiate selectively between infectious and heat and chlorine inactivated poliovirus. PMA RT-PCR was able to differentiate selectively between infectious and noninfectious murine norovirus only when inactivated by chlorine. However, PMA RT-PCR could not differentiate infectious Norwalk virus from virus particles rendered non-infectious by any treatment. PMA RT-PCR assay was not able to differentiate between infectious and UV inactivated viruses suggesting that viral capsid damage may be necessary for PMA to enter and bind to the viral genome. PMA RT-PCR on naked MNV-1 and Norwalk virus RNA suggest that PMA RT-PCR can be used to detect intact, potentially infectious MNV-1 and Norwalk viruses and can be used to exclude the detection of free viral RNA by PCR assay.

Key Words: Propidium monoazide, UV, enteric virus, norovirus

1. **Introduction**

From 1971 to 2010 there have been 888 documented waterborne outbreaks associated with drinking water in the United States, 8% of which were caused by viral pathogens (Brunkard et al., 2011; Hilborn et al., 2013; Liang et al., 2006; Reynolds et al., 2008; Yoder et al., 2008). More than 40% of the waterborne outbreaks resulting in acute gastrointestinal illness were of undetermined etiology, and the characteristics of these outbreaks often were consistent with a viral etiology (Reynolds et al., 2008). Many human enteric viruses such as enteroviruses, noroviruses, rotaviruses, hepatitis A, and adenoviruses are released into the feces of infected individuals and subsequently reach water sources through contamination of water. These viruses can cause a variety of illnesses, such as gastroenteritis, hepatitis, paralysis, aseptic meningitis, herpangina, respiratory illness, fevers, myocarditis, etc (Bosch, 1998; Khetsuriani et al., 2006). Among the enteric viruses, noroviruses (NoVs) are the leading cause of acute viral gastroenteritis in the United States and worldwide (Mead et al., 1999; Siebenga et al., 2009). NoVs can cause disease both in children and adults and are transmitted primarily through the fecal-oral route, either by person-to-person contact or by consumption of contaminated food and water. Waterborne disease outbreaks associated with NoVs have been reported around the world (Hewitt et al., 2007; Hoebe et al., 2004; Maunula et al., 2005; Nygard et al., 2003; Parshionikar et al., 2003) and NoVs are the predominant cause of recent viral waterborne outbreaks in the United States (Blackburn et al., 2004; Liang et al., 2006; Yoder et al., 2008).

The Safe Drinking Water Act (SDWA) requires the U. S. Environmental Protection Agency (EPA) to identify and publish the drinking water Contaminant Candidate List (CCL) periodically, which consists of unregulated and emerging contaminants. Due to the occurrence of enteric viruses in contaminated water and their potential public health impact, several enteric

viruses, including enteroviruses and caliciviruses, are on the CCL 3 (http://www.epa.gov/ogwdw/ ccl/ccl3. html). Enteroviruses and NoVs also are being monitored under EPA's Unregulated Contaminant Monitoring Rule round 3 in a subset of national public systems using groundwater (http://water.epa.gov/lawsregs/rulesregs/sdwa/ucmr/ucmr3/methods.cfm). Within the *Caliciviridae* family, NoVs of the genus *Norovirus* are the primary viruses of concern for drinking water. NoVs have a single stranded positive sense RNA genome and are divided into five genogroups and 32 genetic clusters (Zheng et al., 2006; Zheng et al., 2010). The strains relevant to human disease belong to genotypes within genogroups I, II, and IV (Zheng et al., 2010).

The current method for the detection of infectious viruses in drinking water uses cell culture to determine the infectivity of viruses. There are some promising developments towards the cultivation of NoVs (Jones et al., 2014; Katayama et al., 2014; Taube et al., 2013), but at present there is no established cell line or small animal model that allows for routine detection of infectious human NoVs. In absence of an infectivity assay, molecular methods such as reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (RT-qPCR) are used for the detection NoVs in water. Because these methods detect both infectious and non-infectious virus, the conclusions that may be drawn regarding the public health significance of positive findings generally are considered to be limited. Thus it is important to know whether the detected virus is infectious or non-infectious when conducting a public health risk analysis of virus exposure through consumption of water and when developing strategies to control viral exposure (Reynolds et al., 1996). This general consensus on the relationship of positive PCR finding and public health may not hold up for exposure to untreated groundwaters. A recent

study found that AGI in communities in Wisconsin increases in proportion to the level of NoVs in the groundwater as measured by RT-qPCR in all ages, but especially in children (Borchardt et al., 2012). There was also a relationship between AGI and enterovirus levels detected by RT-qPCR in the communities' drinking water. Nevertheless, it still would be of great value to be able to measure viral infectivity directly using PCR.

Animal viruses, such as feline calicivirus (FCV) and murine norovirus 1 (MNV-1), have been used as surrogates to study NoVs infectivity (Bae and Schwab, 2008; Gibson and Schwab, 2011; Hirneisen and Kniel, 2013; Huang et al., 2000). However, FCV is an oral and respiratory pathogen of cats and belong to the genus *Vesivirus*. MNV-1 is an enteric pathogen of mice that belongs to the *Norovirus* genus, can survive under gastric pH levels (Cannon et al., 2006), is morphologically and genetically similar to human NoVs, and can routinely be grown in cell culture (Wobus et al., 2006), thus making it useful model for NoVs infectivity studies.

Propidium monoazide (PMA) and ethidium monoazide (EMA) are similar photo-reactive DNA intercalating dyes that covalently bind to DNA upon exposure to intense visible light. Unlike EMA which can penetrate live cells, PMA is a membrane impermeable dye that can only penetrate dead cells through compromised cell membranes (Nocker et al., 2006). PMA enters cells with compromised membranes and intercalates into the DNA. Upon exposure to light, the PMA covalently binds to the DNA, resulting in either the removal of bound DNA during the DNA extraction process or chemical alteration of DNA, which prevents the DNA from being amplified by PCR. When live microorganisms with intact cell membrane are exposed to PMA and light, the dye cannot penetrate the cell membrane; thus no DNA modification occurs and DNA can subsequently be amplified by PCR. PMA treatment along with PCR has been used to distinguish between dead and live bacteria (Nocker et al., 2006; Nocker et al., 2007; Pan and

Breidt, 2007), fungi (Vesper et al., 2008), *Giardia* cysts (Sauch et al., 1991), *Cryptosporidium* oocysts (Brescia et al., 2009).

PMA and EMA also have been examined for effectiveness with virus particles (Coudray-Meunier et al., 2013; Graiver et al., 2010; Kim and Ko, 2012; Parshionikar et al., 2010; Sanchez et al., 2012). Graiver and colleagues (Graiver et al., 2010) reported that EMA does not inhibit RT-PCR amplification of non-infectious avian influenza viruses. However, PMA has been shown to distinguish between infectious and non-infectious poliovirus (Parshionikar et al., 2010), MS2 (Kim and Ko, 2012), hepatitis A, and rotavirus (Coudray-Meunier et al., 2013; Sanchez et al., 2012).

The majority of the human enteric viruses are non-enveloped viruses containing a protein capsid that protects the genome. For PMA (or EMA) to be effective, it must be able to penetrate the capsid and bind to the genome, i.e., effectiveness for viruses requires a damaged capsid analogous to the requirement for a damaged bacterial membrane (Nocker et al., 2006). This hypothesis predicts that PMA will only be able to distinguish between infectious and non-infectious viral particles when the inactivation conditions result in a sufficiently damaged capsid. Effective damage could be due to physical loss of capsid protein or to conformational changes the expose the nucleic acid (Cliver, 2009). Due to the importance of human NoVs in waterborne disease, the present study uses MNV-1 as a model to evaluate PMA for selective differentiation of infectious and non-infectious NoVs. Moreover, the effectiveness of PMA to distinguish between infectious and UV inactivated NoVs and poliovirus was evaluated.

2. Materials and Methods

2.1. Viral stock preparation

Poliovirus 1 (chat strain, American Type Culture Collection, Number VR-1562), murine norovirus 1 (MNV-1), and Norwalk virus were used in this study. MNV-1 was provided by Herbert Skip Virgin, Washington University, St. Louis, MO, and Norwalk virus was extracted from a stool specimen from a previous volunteer study that was provided by Gary Richards (Charleston Laboratory, National Marine Fisheries Service). Poliovirus 1 was grown in the laboratory by inoculation onto confluent monolayers of Buffalo green monkey kidney (BGM) cells. After cytopathic effect (CPE) was observed, the cell culture was freeze thawed three times and centrifuged for 15 min at 1,700 x *g* at 4°C. The supernatant was collected and filtered through a 0.22 µm acrodisc 37-mm syringe filter (Pall Corporation, Ann Arbor, MI) pretreated with 1.5% beef extract. MNV-1 was grown in RAW 264.7 cells as described by Bae and Schwab (Bae and Schwab, 2008), with minor modifications. Briefly, MNV-1 was inoculated onto monolayer of RAW 264.7 cells and after virus adsorption, maintenance medium containing Dulbecco's modified Eagle's medium (DMEM; Cellgro, Mediatech, VA) supplemented with 2% low endotoxin fetal bovine serum (FBS; HyClone, Logan, UT), 1% Glutamine, 1% HEPES, and 1% penicillin-streptomycin was added and the flask was incubated at 37°C in 5% CO_2. After CPE was observed, the cell culture was freeze thawed three times and equal volume of Vertrel XF (Dupont, Wilmington, DE) was added to the virus containing medium, mixed by shaking, and was then centrifuged for 15 min at 5,000 x g at 4^oC. The supernatant was collected and filtered through a 0.22 µm acrodisc 37-mm syringe filter (Pall Corporation, Ann Arbor, MI) that

was pretreated with 1.5% beef extract. Poliovirus and MNV-1 stocks were stored at -70°C and thawed shortly before each experiment. Norwalk virus was extracted from stool samples by 10-fold dilution in phosphate-buffered saline containing 0.1% bovine serum albumin (U.S. Biochemical Corp., Cleveland, OH), mixed with an equal volume of Vertrel XF and then centrifuged for 15 min at 5,000 X g at 4°C. The supernatant was collected and filtered through a 0.22 µm acrodisc filter that had been pretreated with 1.5% beef extract. Extracted Norwalk virus was stored at 4°C.

2.2. Plaque assay

The titer and infectivity of poliovirus and MNV-1 after inactivation experiments were determined using plaque assays. For poliovirus 0.5 ml sample was inoculated per 25-cm^2 flask of BGM cells , and then overlaid with agar according to Dahling and Wright (Dahling and Wright, 1986). Flasks were incubated at 37° C and observed for 1 week for plaque formation. For MNV-1, plaque assay was done as described by Wobus et al, (Wobus et al., 2004) with minor modifications. RAW 264.7 cells were seeded into six-well plates at a density of 1 X 10^6 to 1 X 10^7 viable cells per well in cell growth medium (DMEM supplemented with 10% low endotoxin FBS, 1% Glutamine, 1% HEPES, and 1% penicillin-streptomycin. Plates were incubated at 37°C and 5% CO_2 for two days to produce a confluent monolayer. Tenfold dilutions of MNV-1 were prepared in maintenance medium, the cell growth medium from the wells were aspirated off, and

0.5 ml of each viral dilution was inoculated into duplicate wells. Plates were incubated for an hour at room temperature on a rocking apparatus. After the incubation period, the inoculum was aspirated off and the cells were overlaid with 2 ml of 1.5% SeaPlaque agarose (Lonza, Rockland, ME) in minimum essential medium (MEM) supplemented with 10% FBS, 1% Glutamine, 0.5% HEPES, and 1% penicillin-streptomycin (complete MEM). Plates were incubated at 37°C and 5% CO_2 for 24 h. To visualize the plaques, 2 ml of a second overlay medium containing 1.5 % SeaKem agarose (Lonza) in complete MEM and 1% neutral red was added in each well. Plates were incubated for an additional 24 h and then plaques were counted. Virus titer was recorded as PFU/ml.

2.3. Determination of stock titer of Norwalk virus

The titer of the stock of Norwalk virus was determined by RT-PCR (see below) using a 10-fold dilution series using 10 replicates of each dilution and the RT-PCR units was calculated using the MPNV calculator (an updated version of this program is available from http://www.epa.gov/nerlcwww/microbes/epamicrobiology.html).

2.4. Virus inactivation by heat

Poliovirus 1 and Norwalk virus were heat inactivated at 72^{o}C. MNV-1 was heat inactivated at 72^{o}C and 99^{o}C. Viruses were diluted in phosphate-buffered saline (PBS; pH 7) at a targeted concentration of about 10^3 plaque forming units (PFU)/mL (poliovirus and MNV-1) or 10^3 RT-PCR unit/ml (Norwalk virus) for inactivation experiments. For inactivation at 72^{o}C, 50-

ml conical tubes containing 4.5 ml PBS was preheated to 72^{o}C in a water bath and then 0.5 ml of 10^4 PFU or RT-PCR units/ml viruses were added to achieve a concentration of 10^3 PFU or RT-PCR units/ml. A temperature control tube was used in the water bath to monitor the temperature inside the 50-ml tubes. Viruses were incubated at 72°C for 5, 6, and 7 seconds or 5 minutes and were chilled immediately by placing the tubes on ice. After heat treatment, inactivated poliovirus and MNV-1 were stored at -70^{o}C and Norwalk virus was stored at 4^{o}C. For inactivation at 99^{o}C, MNV-1 was diluted in 100 µl of PBS at a targeted concentration of 10^3 PFU/ml and were heat inactivated for 5, 10, 15, 20, 25, and 30 minutes using a PCR thermal cycler (DNA Engine PTC-200 Thermal Cycler; BIO-RAD, Hercules, CA). Poliovirus and MNV-1 infectivity were determined by plaque assay. No plaques were observed in virus samples heat treated at 72^{o}C for 5 min, so virus infectivity after 99^{o}C heat treatment was not measured. Since there is no cell line available for the propagation of Norwalk virus, inactivation and thereby loss of infectivity was assumed based on the results of MNV-1 inactivation. A suspension of viruses without exposure to heat was used as a positive process control.

2.5. Virus inactivation by chlorine.

Poliovirus 1, MNV-1, and Norwalk virus were diluted in chlorine-demand-free buffer (CDF; pH 7) at a targeted concentration of 10^3 plaque forming units (PFU)/mL (poliovirus and MNV-1) or 10^3 RT-PCR unit/ml (Norwalk virus). Virus samples were placed in a 5± 2^{o}C water bath and chlorine was added to achieve an initial free chlorine concentration of approximately 0.5mg/L. Free and total chlorine were measured by the DPD (N,N Diethyl-1,4 Phenylenediamine Sulfate) method using colorimetric method with a colorimeter (Hach Company, Loveland, CO). Ten ml aliquots were removed at 0.5, 1, 2, 5, 10 and 20 minutes and added to tubes containing

0.2 ml of 10% sodium thiosulfate to neutralize remaining free chlorine. As a process control, a suspension of viruses without exposure to chlorine was prepared and was used as a positive control in PMA experiments. For each virus, triplicate experiments were conducted for chlorine inactivation. After chlorine treatment, inactivated poliovirus and MNV-1 were stored at -70^{o}C and Norwalk virus was stored at 4^{o}C. Poliovirus and MNV-1 inactivation at each time point were determined using plaque assay. For Norwalk virus, loss of infectivity was assumed based on the results of MNV-1 inactivation by plaque assay. The samples for the first time point at which no plaques were observed and samples from the 20 min time point were used for subsequent PMA assays.

2.6. Virus inactivation by Ultraviolet Light (UV)

UV disinfection of viruses was done using a low pressure (254 nm UV light) collimated beam apparatus described by Simonet et al. (Simonet and Gantzer, 2006). UV intensity delivered by the collimated beam was measured using a calibrated radiometer. Poliovirus 1, MNV-1, and Norwalk virus were diluted in phosphate-buffered saline (PBS; pH 7) at a concentration of 10^3 plaque forming units (PFU)/mL (poliovirus and MNV-1) or 10^3 RT-PCR unit/ml (Norwalk virus). Virus samples were transferred to petri dishes and were placed on a magnetic stir plate for continuous slow stirring of the viral suspension. The average irradiance in the virus suspension was determined by the UV absorbance of the test suspensions at the wavelength of 254 nm, the sample depth, and the incident average irradiance. Exposure times were calculated by dividing the desired UV fluence by the average UV irradiance. For uniform lamp output, the lamp was warmed for at least 30 minutes before each UV treatment. Two UV doses, 33 mJ/cm^2 and 187 mJ/cm^2 were evaluated for each virus inactivation. As a process control, a suspension of viruses

without exposure to UV light was prepared and was used as a positive control in PMA experiments. For each virus and each UV dose, three replicate inactivation experiments were conducted. After the completion of the UV treatment, inactivated poliovirus and MNV-1 were stored at -70^{o}C and Norwalk virus was stored at 4^{o}C. For poliovirus and MNV-1, UV inactivation was determined by plaque assay. For Norwalk virus, loss of infectivity was assumed based on the results of MNV-1 inactivation by plaque assay.

2.7. Propidium monoazide treatment

Both infectious and inactivated viruses were subjected to PMA treatment. PMA (Biotium, Hayward, CA) was reconstituted with 20% dimethyl sulphoxide (DMSO; Sigma, St. Louis, MO) to a concentration of 1 mg/mL (1.95 mM) and stored at -20°C. In a room with minimal light, 25 µL of stock PMA was added to 100 µL of infectious or inactivated virus samples containing 100 PFU of poliovirus or MNV-1 or 100 RT-PCR units of Norwalk virus in a 1.5 ml microcentrifuge tube. Final concentration of PMA was adjusted to 348 µM by adding 15 µl of nuclease free water. Samples were mixed by pulse vortexing and incubated at room temperature for 5 min. After incubation, the tubes were placed sideways on ice to prevent overheating and then were exposed to an 800 watt light at a distance of 20 cm for 3 minutes. After light exposure, room lights were turned on and viral RNA was extracted. Viruses treated with PMA but without the exposure of light were used as a control.

2.8. Virus RNA extraction

Viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA)

using the spin column protocol described by the manufacturer. Extracted RNA was stored at -20°C and was thawed before RT-PCR/RT-qPCR assay.

2.9. RT-PCR

Primers for poliovirus, MNV-1, and Norwalk virus are presented in Table 1. Each RT reaction was performed by adding 5 µl of viral RNA to a mix containing 3 µl of 10x PCR buffer II, 1.5 mM $MgCl_2$, 0.67 mM of each deoxyribonucleotide triphosphate, 50 pmol of downstream primers, 30 U of recombinant RNasin (Promega, Madison, WI) and 50 U of murine leukemia virus reverse transcriptase (Thermo Fisher Scientific, Grand Island, NY) in a final reaction volume of 30 µl and cDNA was prepared by incubating at 43°C for 60 min. Reverse transcriptase then was inactivated by a step at 94°C for 5 min. PCR was performed by adding 20 µl of a mix containing 5 µl of 10x PCR buffer (, 4.5 mM $MgCl_2$, 50 pmol of upstream primer, and 5 U of AmpliTaq Gold polymerase (Thermo Fisher Scientific, Grand Island, NY). Viral cDNA was amplified with 40 cycles of 95°C for 1 min, 52°C for 1 min and 30 s, and 72°C for 2 min for poliovirus; 94°C for 30 s, 56°C for 1 min and 30 s and 72°C for 1 min for MNV-1; and 94°C for 30 s, 50°C for 1 min and 30 s and 72°C for 1 minute for Norwalk virus. Following the 40 cycles, samples were incubated at 72°C for 15 min for poliovirus and 7 min for MNV-1 and Norwalk virus and then kept at 4°C or at -20°C for long-term storage. The quality assurance guidelines described by the U.S. Environmental Protection Agency (Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples, 2004) were followed during PCR analysis of the samples.

2.10. Agarose gel electrophoresis

Ten microliters of the RT-PCR product was added with 2 µl of bromophenol blue and 8

µl of nuclease free water to increase the volume to 20 µl, needed for loading in the E-gel (2 % agarose, double comb; Invitrogen, Carlsbad, CA) wells. Two µl of 100 bp ladder was mixed with 18 µl of nuclease free water and the 20 µl mix was loaded in a well. PCR amplicons were visualized using the Kodak Gel Logic 100 Imaging system (Eastman Kodak Company, Rochester, NY).

2.11. RT-qPCR

Two step RT-qPCR was performed using the Applied Biosystems 7900HT system (Applied Biosystems, Foster City, CA). Primers and probes are presented in Table 1. Each RT reaction was performed by adding 5 µl of viral RNA to a mix containing 2.5 µl of 10x PCR buffer II, 1.5 mM $MgCl_2$, 0.8 mM of each deoxyribonucleotide triphosphate, 20 pmol of downstream primers, 24 U of recombinant RNasin (Promega, Madison, WI) and 40 U of murine leukemia virus reverse transcriptase (Thermo Fisher Scientific, Grand Island, NY) in a final reaction volume of 25 µl and cDNA was prepared by incubating at 43°C for 60 min. Reverse transcriptase subsequently was inactivated by a step at 94°C for 5 min. PCR was done by adding 25 µl of a mix containing 2.5 µl of 10x PCR buffer II (Thermo Fisher Scientific, Grand Island, NY), 8.5 mM $MgCl_2$, 1 µl ROX reference dye, 20 pmol of forward primer, 5 pmol probe, and 2.5 U of AmpliTaq Gold polymerase (Thermo Fisher Scientific, Grand Island, NY). Amplification was done with a hot start polymerase activation step for 10 min at 95^{o}C, followed by 40 cycles of 15 s at 95^{o}C and 1 min at 60^{o}C. Each reaction was conducted in duplicate.

2.12 Statistical Analysis

Fold reductions were calculated from the difference between the mean Cq value of an experimental test (Cq_{exp}) and the mean Cq value of the untreated infectious control (Cq_{con}) using

1 the formula, Fold reduction = $2^{(Cqexp - Cqcon)}$. Statistical comparisons were performed by One Way
2 Anova and the Holm-Sidak method for all pairwise multiple comparisons using SigmaPlot
3 (version 12.5, Systat Software, Chicago, IL).

3. Results

3.1. Virus inactivation by heat, chlorine, and UV

Poliovirus and Norwalk virus were inactivated by heat at 72^{o}C for 5, 6, and 7 seconds and for 5 minutes. MNV-1 was inactivated at 72^{o}C for 5 minutes. Loss of infectivity for poliovirus and MNV-1 were measured by plaque assay. Both poliovirus and MNV-1 lost their infectivity at all 72^{o}C heat treatments (Table 2). Poliovirus and MNV-1 both lost infectivity when exposed to low (33 mJ/cm^{2}) and high (187 mJ/cm^{2}) UV doses (Table 2). The mean CT (the concentration of free chlorine multiplied by time of contact with virus) values for 3 log inactivation of poliovirus and MNV-1 by chlorine were 5.3 mg-min/L and 0.75 mg-min/L, respectively. Because Norwalk virus infectivity could not be measured by cell culture, MNV-1 was used as a model for Norwalk virus inactivation. Norwalk virus was assumed to have lost its infectivity with treatments that inactivated MNV-1.

3.2. PMA RT-PCR on infectious and heat inactivated viruses

Infectious and heat inactivated poliovirus were treated with PMA (348 µm), exposed to light, RNA was extracted, and standard RT-PCR or RT-qPCR were performed. Primers for both RT-PCR and RT-qPCR (Table 1) targeted the 5$^{/}$ untranslated region of the genome and produced amplicon sizes of 196 bp and 142 bp, respectively. PMA treatment of infectious poliovirus with or without light exposure did not have any effect on the RT-PCR detection of infectious viruses (Figure 1). When non-infectious poliovirus (inactivated by 72^{o}C for 5, 6, or 7 seconds) was treated with PMA, the expected amplicon (196 bp) was observed in treatments without light exposure but not in PMA treated viruses exposed to light. The RT-qPCR assay of infectious and heat inactivated poliovirus produced results similar to RT-PCR (Table 3).

Treatment of infectious poliovirus with or without light exposure after PMA treatment or treatment of non-infectious poliovirus with PMA but without light exposure resulted in no virus reduction, while treatment of poliovirus rendered non-infectious by treatment at 72ºC for as short as 5 seconds with PMA and light resulted in a statistically significant >275-fold decrease in virus (P= <0.001). These data suggest that PMA treatment and light exposure prior to PCR can differentiate between infectious and non-infectious poliovirus by RT-PCR or RT-qPCR.

PMA treatment with or without the exposure of light could not differentiate infectious and heat inactivated MNV-1 (Figure 2) and Norwalk virus (data not shown) by RT-PCR. PMA treatment partially distinguishes between infectious and heat inactivated MNV-1 when measured by RT-qPCR, with a statistically significant 6-fold reduction of non-infectious virus when exposed to light (Table 4, P= <0.001). Treatment also resulted in a very small 2-fold reduction of heat inactivated Norwalk virus by RT-qPCR (Table 4). To investigate whether higher temperature treatment is required for MNV-1 and Norwalk virus viral capsid damage, MNV-1 was treated at 99°C for varying times of 5, 10, 15, 20, 25, and 30 minutes, treated with PMA with or without light exposure, and after RNA extraction, PCR was performed. Although PMA treatment with light exposure reduced the band intensity, it did not eliminate PCR detection of MNV-1 completely (data not shown).

3.3. PMA RT-PCR on infectious and chlorine inactivated viruses

To evaluate whether PMA treatment can differentiate between infectious and chlorine inactivated viruses, infectious and chlorine inactivated viruses were treated with PMA, exposed to light, RNA was extracted, and standard RT-PCR was performed. PMA-RT-PCR could not differentiate between infectious and inactivated MNV-1 when virus was inactivated using a low

CT (0.06 mg-min/L) chlorine level, but was able to differentiate (2 out of 3 replicates) or partially differentiate (1 replicate) between infectious and chlorine inactivated viruses when the viruses were inactivated at the higher CT (2.7 mg-ml/min) chlorine level (Figure 3). However, the PMA RT-PCR assay could not differentiate between infectious and chlorine inactivated Norwalk virus (data not shown).

3.4. PMA RT-PCR on infectious and UV inactivated viruses

Viruses were inactivated using two UV doses (32 mJ/cm^2 and 187 mJ/cm^2) and poliovirus and MNV-1 inactivation were confirmed by plaque assay. Norwalk virus was assumed to have lost its infectivity with UV doses that inactivated MNV-1. Infectious and UV inactivated viruses were treated with PMA, exposed to light, RNA was extracted, and standard RT-PCR was performed. PMA treatment of infectious and UV inactivated poliovirus with or without light exposure was not able to differentiate between infectious and non-infectious viruses (data not shown). Thus, there was no effect of PMA. Similar data was observed for MNV-1 (Figure 4) and Norwalk virus (not shown). These results indicate that although exposure to UV light ceased virus replication in cells, PMA could not enter the inactivated viruses to bind with viral RNA. Thus there were no effect of PMA treatment to differentiate between infectious and UV inactivated viruses.

3.5. PMA RT-PCR on naked viral RNA

Because PMA RT-PCR produced differential results on viruses inactivated by different conditions, PMA binding ability with naked viral RNA (extracted using Qiagen RNA extraction kit) was evaluated. Also, the use of a higher concentration of PMA (696 µm final concentration)

and longer time light exposure (5 min) were evaluated. Infectious virus and viral RNA were treated with PMA with and without light exposure and RNA was extracted prior to RT-PCR assay. The expected size of amplicons for MNV-1 (159 bp; Figure 5) and for Norwalk virus (213 bp; not shown) were observed when viral RNA was treated with either 348 or 696 µm PMA without light exposure. No amplicon was observed when MNV-1 RNA was treated with 696 µm PMA and exposed to light for either 3 or 5 minutes (Figure 5), but low-level amplification was observed in some replicates using 348 µm PMA treatment and light. For Norwalk virus no amplicon was observed when viral RNA was treated with 348 µm or 696 µm PMA treatment and there was no difference whether the light exposure was for 3 or 5 minutes (not shown). Similar data was observed for RT-qPCR (Table 5). An increase of PMA concentration to 696 µm caused an 20-fold reduction in infectious virus when exposed to 5 minutes of light treatment, but treatment of RNA at either PMA concentration or light treatment prevented detection of viral RNA, representing a statistically significant >1382-fold reduction. These results indicate that upon exposure to light PMA binds with both MNV-1 and Norwalk virus genomes, rendering them undetectable by RT-PCR and RT-qPCR.

4. **Discussion**

PMA is known to covalently bind to DNA/RNA upon exposure to intense visible light. A number of studies have exploited the PMA binding mechanism to differentiate between live and dead bacteria, fungi, or parasites (Brescia et al., 2009; Nocker et al., 2006; Nocker et al., 2009; Nocker et al., 2007; Pan and Breidt, 2007; Vesper et al., 2008), or between infectious and non-infectious viruses (Coudray-Meunier et al., 2013; Fittipaldi et al., 2010; Graiver et al., 2010; Kim and Ko, 2012; Parshionikar et al., 2010; Sanchez et al., 2012). PMA effectiveness for distinguishing between infectious poliovirus, Norwalk virus, and MNV-1 and virus particles inactivated by heat, chlorine, and UV were examined in this study. MNV-1 was used as a model virus for NoVs because the latter cannot be grown in cell culture.

Poliovirus was inactivated by heat at 72^{o}C in as little as 5 seconds, which is similar to the data observed by Nuanualsuwan and Cliver (Nuanualsuwan and Cliver, 2002). MNV-1 and Norwalk virus were treated at 72^{o}C for 5 min and at this temperature MNV-1 completely lost its infectivity in cell culture. Norwalk virus was assumed to have lost its infectivity upon exposure to the same treatment as MNV-1.

The CT value for chlorine inactivation for 3-log reduction of poliovirus was 5.3 mg-min/L compared to 2.15 mg-min/L reported by Nuanualsuwan and Cliver (2002). At a CT value of 5.3 mg-min/L poliovirus completely lost its infectivity. No detectable infectivity for MNV-1 was observed at CT of 0.75 mg-min/L. The actual CT for three log inactivation of MNV-1 could be lower, but contact times shorter than 0.5 min were not evaluated. Previous studies reported that CT values of <0.02 and 0.25 mg-min/L provided a 3 log and 4.1 log reduction of MNV-1, respectively (Cromeans et al., 2010; Kitajima et al., 2010). Both UV doses (33 and 187 mJ/cm^2) were sufficient to inactivate poliovirus and MNV-1. Previous studies reported 3 log reduction of

poliovirus by UV dose of 23 mJ/cm^2 (Gerba et al., 2002) and 3.3 log reduction of MNV-1 by UV dose of 25 mJ/cm^2 (Lee et al., 2008). A UV dose of 187 mJ/cm^2 was tested to evaluate whether a high UV dose applied to inactivate viruses can damage the viral capsid.

The PMA RT-PCR assay was able to detect infectious, but not heat (72^{o}C) inactivated poliovirus. The RT-qPCR assay of infectious and heat inactivated poliovirus produced results similar to RT-PCR. PMA treatment also was able to differentiate between infectious and chlorine inactivated poliovirus. These data are further confirmation of previous findings that PMA RT-PCR assay can differentiate between infectious and inactivated enteroviruses (Parshionikar et al., 2010). Very similar findings have been reported for infectious and heat-inactivated hepatitis A virus (Sanchez et al., 2012). This virus belongs to the same picornavirus family and has a similar structure to poliovirus.

The PMA RT-PCR assay was not able to differentiate between infectious and heat inactivated MNV-1 and Norwalk virus, but the PMA RT-qPCR assay demonstrated small reductions in detectable non-infectious virus. Similar results also were reported for MNV using heat inactivation at 72°C for 10 min and either 125 or 250 µm PMA (Kim and Ko, 2012). The differential ability of PMA-RT-PCR assay to distinguish between infectious and non-infectious viruses inactivated using different methods may be a function of capsid damage occurring during inactivation. The viruses evaluated in the study are non-enveloped RNA viruses and their genomes are enclosed within a protective protein capsid. The capsid structure of poliovirus is different from MNV-1 and Norwalk virus and the extent of the capsid damage by heat may be different for these viruses. Poliovirus capsid consists of 60 subunits which contain four viral polypeptides VP1, VP2, VP3, and VP4, arranged with icosahedral symmetry (Pallansch et al., 1984), whereas MNV-1 and Norwalk virus capsid consists of 90 dimers of a single major viral

protein (Jiang et al., 1992; Prasad et al., 1999; Shoemaker et al., 2010). It is plausible that although heat treatment causes loss of infectivity in MNV-1 and Norwalk virus, their capsids remain more protective than those of enteroviruses, thus blocking PMA entry. Surprisingly, partial protection was observed even upon heating MNV-1 at 99°C, as this temperature has been used to release RNA from enteric viruses prior to PCR (Fout et al., 2003; Parshionikar et al., 2004). This suggests either that at this temperature some of the viral capsid remained undamaged or that the denatured capsid protein is able to protect a fraction of the genome and prevent PMA binding. A recent study by Fittipaldi et al. (Fittipaldi et al., 2010) reported that PMA-qPCR could not differentiate between infectious bacteriophage T4 and virus heat inactivated at 85^{o}C or by use of a proteolytic enzyme. However, when bacteriophage T4 was inactivated at a temperature of 110^{o}C, PMA-qPCR was able to discriminate between infectious and non-infectious viruses. This suggests that either the higher temperature damaged the capsid sufficiently to allow PMA to penetrate and bind to the phage DNA (Fittipaldi et al., 2010) or that it resulted in the release of the DNA.

If PMA is effective only with viruses when inactivation leads to capsid damage, it should not be or be less effective on chlorine and UV-inactivated viruses. The primary damage due to these treatments is on viral nucleic acid (Eischeid and Linden, 2011; Li et al., 2002), but protein damage can occur at high concentrations or doses. When low chlorine CT was applied for MNV-1 inactivation, PMA RT-PCR could not differentiate between infectious and chlorine inactivated MNV-1, but it could differentiate between infectious and chlorine inactivated MNV-1 when MNV-1 was inactivated using a higher chlorine CT. These results suggest a higher chlorine CT is required for MNV-1 capsid damage and subsequent PMA entrance and binding. In addition, PMA RT-PCR results of UV inactivated viruses (poliovirus, MNV-1, and Norwalk

virus) from this study also support this hypothesis. Although the applied UV doses caused infectivity loss in poliovirus and MNV-1, PMA RT-PCR could not differentiate between infectious and UV inactivated viruses, even at the higher (187 mJ/cm^2) UV dose applied. Eischeid and Linden recently examined UV damage on adenovirus proteins (Eischeid and Linden, 2011). They showed that low-pressure UV, such as used here, results in very little loss of a number of viral proteins at doses less than 186 mJ/cm^2. Greater losses occurred with medium pressure UV at all doses, with major losses occurring at a medium pressure dose of 300 mJ/cm^2. Future experiments should be performed using medium pressure UV to evaluate whether PMA can distinguish between infectious and inactivated virus particles under these conditions.

Another possible explanation for ineffectiveness of the PMA assay with heat-inactivated MNV-1 could have been that PMA does not bind well to the viral RNA that is targeted by the primers used in this study; however, this explanation can be ruled out because PMA treatment of extracted RNA prevented detection (Figure 7, Table 5). When naked MNV-1 and Norwalk virus RNA were used in PMA RT-PCR, viral RNA could not be detected by RT-PCR after PMA treatment and light exposure. These data clearly indicate that PMA can bind to both MNV-1 and Norwalk virus RNA and render it undetectable upon exposure to light. These data do not rule out the possibility that PMA may bind to certain regions of the genome preferentially. The choice of the PCR target region or size may be important (Coudray-Meunier et al., 2013), and could lead to different results between research groups.

In conclusion, the differential ability of PMA-RT-PCR assay to distinguish between infectious and non-infectious viruses seems to be a function of capsid damage occurring during inactivation, although for some viruses, it could be the ability of PMA to bind

to the viral RNA. PMA RT-PCR can exclude the detection of naked MNV-1 and Norwalk virus RNA and can be used to detect potentially infectious NoVs selectively. Also, PMA RT-PCR can be used for assessing damage of viral capsid by different inactivation mechanism.

Highlights:

- PMA RT-PCR was able to differentiate selectively between infectious and heat and chlorine inactivated poliovirus. PMA RT-PCR was able to differentiate between infectious and noninfectious murine norovirus 1 (MNV-1) selectively only when inactivated by chlorine.
- PMA RT-qPCR was able to differentiate between infectious and heat inactivated poliovirus selectively, but not Norwalk virus. A very small differentiation was observed between infectious and heat inactivated MNV-1.
- PMA RT-PCR assay was not able to differentiate between infectious and UV inactivated viruses suggesting that PMA was unable to reach the virus genome.
- PMA RT-PCR and PMA RT-qPCR on naked MNV-1 virus RNA suggest that PMA RT-PCR can be used to detect intact (potentially infectious) viruses selectively and can be used to exclude the detection of free viral RNA by PCR assay.

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Figure 1: RT-PCR of Infectious and Heat-Inactivated Poliovirus 1

Poliovirus was treated with 348 µM PMA and exposed to light for 3 minutes. The virus-positive amplified product is 196 bp.

Gel a: Lane 1 and 9 = 100 bp ladder; Lane 2 and 3 = RT negative; Lane 4 and 5 = PCR negative; Lane 6 and 7 = infectious poliovirus treated with PMA but without the exposure to light; Lane 8 and 10 = infectious poliovirus treated with PMA and exposed to light; Lane 11 and 12 = infectious poliovirus treated with DMSO without exposure to light; Lane 13 and 14 = negative control (no virus) treated with PMA and light; Lane 15 and 16 = negative control (no virus) treated with PMA without light exposure.

Gel b: Lane 1 and 9 = 100 bp ladder; Lane 2, 3, 6, 7, 11, and 12 = non-infectious poliovirus treated with PMA but without the exposure to light; Lane 4, 5, 8, 10, 13, and 14 = non-infectious poliovirus treated with PMA and exposed to light; Lane 15 and 16 =infectious poliovirus without any treatment. Poliovirus was rendered non-infectious by heating to 72ºC for 5 seconds (Lanes 2 to 5), 6 seconds (Lanes 6 to 8, 10), or 7 seconds (Lanes 11 to 14).

Figure 2: PMA RT-PCR of Infectious and Heat-Inactivated Murine Norovirus-1 (MNV-1)

MNV-1 was treated with 348 µM PMA and exposed to light from 3 minutes. The virus-positive amplified product is 159 bp.

Gel a: Lane 1 and 9 = 100 bp ladder; Lane 2 and 3 = RT negative; Lane 4 and 5 = PCR negative; Lane 6 and 7= infectious MNV-1 without any treatment; Lane 8 and 10 = infectious MNV-1 with only light exposure; Lane 11 and 12 = infectious MNV-1 treated with PMA and light; Lane 13 and 14= infectious MNV-1 treated with PMA without the exposure to light; Lane 15 and 16= negative control (no virus) treated with PMA and light.

Gel b: Lane 1 and 9 = 100 bp ladder; Lane 2 and 3 = negative control (no virus) treated with PMA without light; Lane 4, 5, 8, 10, 13, and 14 = non-infectious MNV-1 treated with PMA and light; Lane 6, 7, 11, 12, 15, and 16 = non-infectious MNV-1 treated with PMA without the exposure to light. MNV-1 was rendered non-infectious by heating to 72ºC for 5 minutes.

Figure 3: PMA RT-PCR of infectious and chlorine inactivated MNV-1

Gel a:

Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= RT negative; Lane 4 and 5= PCR negative; Lane 6 and 7= negative control (no virus) treated with PMA without light exposure; Lane 8 = empty; Lane 10 and 11= infectious MNV-1; Lane 12 and 13= infectious MNV-1 treated with PMA without the exposure to light; Lane 14 and 15= infectious MNV-1 treated with PMA and light; Lane 16= empty.

Gel b: (chlorine inactivated at CT value 0.06 mg/l)

Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= non-infectious MNV-1 treated with PMA without exposure to light; Lane 4 and 5= non-infectious MNV-1 treated with PMA and light; Lane 6 and 7= non-infectious MNV-1 treated with PMA without exposure to light; Lane 8=empty; Lane 10 and 11= non-infectious MNV-1 treated with PMA and light; Lane 12 and 13= non-infectious MNV-1 treated with PMA without exposure to light; Lane 14 and 15= non-infectious MNV-1 treated with PMA and light; Lane 16=empty.

Gel c: (chlorine inactivated at CT value 2.7 mg/l)

Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= non-infectious MNV-1 treated with PMA without exposure to light; Lane 4 and 5= non-infectious MNV-1 treated with PMA and light; Lane 6 and 7= non-infectious MNV-1 treated with PMA without exposure to light; Lane 8=empty; Lane 10 and 11= non-infectious MNV-1 treated with PMA and light; Lane 12 and 13= non-infectious MNV-1 treated with PMA without exposure to light; Lane 14 and 15= non-infectious MNV-1 treated with PMA and light; Lane 16=empty.

Figure 4: RT-PCR for infectious and UV disinfected MNV-1.

Gel a:

Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= RT negative; Lane 4 and 5= PCR negative; Lane 6 and 7= negative control (no virus) treated with PMA without light exposure; Lane 8 = empty; Lane 10 and 11= infectious MNV-1; Lane 12 and 13= infectious MNV-1 treated with PMA without the exposure to light; Lane 14 and 15= infectious MNV-1 treated with PMA and light; Lane 16=empty.

Gel b: (UV inactivated at 33 mJ/cm^2)

Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= non-infectious MNV-1 treated with PMA without exposure to light; Lane 4 and 5= non-infectious MNV-1 treated with PMA and light; Lane 6 and 7= non-infectious MNV-1 treated with PMA without exposure to light; Lane 8=empty; Lane 10 and 11= non-infectious MNV-1 treated with PMA and light; Lane 12 and 13= non-infectious MNV-1 treated with PMA without exposure to light; Lane 14 and 15= non-infectious MNV-1 treated with PMA and light; Lane 16=empty.

Gel c: (UV inactivated at 187 mJ/cm^2)

Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= non-infectious MNV-1 treated with PMA without exposure to light; Lane 4 and 5= non-infectious MNV-1 treated with PMA and light; Lane 6 and 7= non-infectious MNV-1 treated with PMA without exposure to light; Lane 8=empty; Lane 10 and 11= non-infectious MNV-1 treated with PMA and light; Lane 12 and 13= non-infectious MNV-1 treated with PMA without exposure to light; Lane 14 and 15= non-infectious MNV-1 treated with PMA and light; Lane 16=empty.

Figure 5: RT-PCR of naked MNV-1 RNA

Gel a: Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= RT negative; Lane 4 and 5= PCR negative; Lane 6 and 7= negative control (no virus) treated with PMA without light exposure; Lane 8 = empty; Lane 10 and 11= infectious MNV-1; Lane 12 and 13= infectious MNV-1 treated with PMA without the exposure to light; Lane 14 and 15= infectious MNV-1 treated with PMA and light; Lane 16=empty.

Gel b (PMA concentration = 348 µm): Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= MNV-1 RNA treated with PMA without exposure to light; Lane 4 and 5= MNV-1 RNA treated with PMA and 3 min. of light exposure; Lane 6 and 7= MNV-1 RNA treated with PMA and 5 min. of light exposure; Lane 8= empty; Lane 10 and 11= MNV-1 RNA treated with PMA without exposure to light; Lane 12 and 13= MNV-1 RNA treated with PMA and 3 min. of light exposure; Lane 14 and 15= MNV-1 RNA treated with PMA and 5 min. of light exposure; Lane 16= empty.

Gel c (PMA concentration = 696 µm): Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= MNV-1 RNA treated with PMA without exposure to light; Lane 4 and 5= MNV-1 RNA treated with PMA and 3 min. of light exposure; Lane 6 and 7= MNV-1 RNA treated with PMA and 5 min. of light exposure; Lane 8= empty; Lane 10 and 11= MNV-1 RNA treated with PMA without exposure to light; Lane 12 and 13= MNV-1 RNA treated with PMA and 3 min. of light exposure; Lane 14 and 15= MNV-1 RNA treated with PMA and 5 min. of light exposure; Lane 16= empty.

Table 1. Sequences of primers and probes used for RT-PCR and RT-qPCR

Primer or Probe name	Virus	Sequence (5′ - 3′)	Product size	Product/probe region	Reference or source
MRD14 (EV-L) [a]	Poliovirus [b]	CCTCCGGCCCCTGAATG	196	5′ untranslated region (444-638)	(De Leon et al., 1991)
MRD13 (EV-R)		ACCGGATGGCCAATCCAA			
MKPOLIO2	Poliovirus	ATGGCCAATGAGCATATGGGACTG	197	(3492-3688)	This study
MKPOLIO1		GTAGAGACCTCTTAGTCACAGAATCAAGAG			
GSF TQM up	Poliovirus	CCCTGAATGCGGCTAAT	143	5′ untranslated region (452-594)	(Parshionikar et al., 2010)
GSF TQM low		TGTCACCATAAGCAGCCA			
GSF TQM probe [c]		ACGGACACCCAAAGTAGTCGGTTC - TAMRA		(535-557)	
MNVKS1	MNV [d]	AGGTCATGCGAGATCAGCTT	159	ORF1, protease, polymerase region (3727-3885)	(Bae and Schwab, 2008)
MNVKS2		CCAAGCTCTCACAAGCCTTC			
MNVKS3 probe		CAGTCTGCGACGCCATTGAGAA - TAMRA		(3799-3820)	
MKMNV1	MNV	CCAGGAGAACGCCATCAATATCAAAAC	434	ORF3, 3'-untranslated region, (6944-7377)	This study
MKMNV2		GCATCTAACTACCACAAAGAAAAGAAAGCAG			
MON 432	Norwalk virus [e]	TGG ACI CGY GGI CCY AAY CA	213	RNA polymerase (5093-5305)	(Beuret et al., 2000; Parshionikar et al., 2003)
MON 434		GAA SCG CAT CCA RCG GAA CAT			
MKNO1	Norwalk virus	GGTATCAACAAAATTTGCAACTGC	368	(7050-7417)	This study
MKNO2		GACCCAACTAGTGGTTCGAGAA			
NoF1 (QN1F4)	Norwalk virus	GGC TGG ATG CGN TTC CAT	86	Polymerase-capsid region (5291-5376)	(da Silva et al., 2007) (Svraka et al., 2007)
NoR1 (NV1LCR)		CCT TAG ACG CCA TCA TCA TTT AC			
NorI1MGB probe		ATY GCG ATC TCC TGT CCA-MGBNFQ		(5321-5338)	This study

[a] If different, primer names in parentheses are those used in the cited references.
[b] Nucleotide sequence from accession number V01149
[c] All qPCR probes labeled with 5'-FAM
[d] Nucleotide sequence from accession number NC_008311
[e] Nucleotide sequence from accession number M87661

Table 2. Inactivation of Poliovirus, and Murine Norovirus by Heat (72°C) and UV as determined by cell culture [a]

		Poliovirus (PFU/ml)				Murine norovirus (PFU/ml)			
Treatment	**Duration of heat exposure or UV dose (mJ/cm^2)**	**Control**	**Rep. 1**	**Rep. 2**	**Rep. 3**	**Control**	**Rep. 1**	**Rep. 2**	**Rep.3**
Heat (72°C)	5, 6, or 7 seconds	4×10^2	0	0	0	ND	ND	ND	ND
	5 minutes	2.5×10^2	0	0	0	9×10^2	0	0	0
UV	33	9×10^2	0	0	0	4×10^2	0	0	0
	187	4×10^2	0	0	0	1.3×10^3	0	0	0

[a] Abbreviations: Rep = replication; ND= Not done

Table 3. Cq values for infectious and non-infectious poliovirus detection by PMA-RT-qPCR with or without the exposure of light [a]

Virus	**PMA**	**Light**	**Avg. Cq**	**Cq Std. Dev.**	**Fold Reduction**
Control (no virus)	+	-	undetermined		
Control (no virus)	+	+	undetermined		
	-	-	31.89	0.51	
Infectious poliovirus	+	-	32.44	0.39	1
	+	+	32.47	0.26	1
Non-infectious poliovirus	+	-	32.42	0.53	1
(72°C, 5 sec)	+	+	>40		>275 [b]
Non-infectious poliovirus	+	-	32.32	0.33	1
(72°C, 6 sec)	+	+	>40		>275 [b]
Non-infectious poliovirus	+	-	31.94	0.70	1
(72°C, 7 sec)	+	+	>40		>275 [b]

[a] Each RT-qPCR reaction was done in duplicate.

[b] There is a statistically significant difference between these values and all other values (P = <0.001; power at alpha = 0.050 of 1.000).

Table 4. Cq values for infectious and 72°C, 5 minute heat inactivated MNV-1 and Norwalk virus detection by PMA RT-qPCR [a]

Virus	PMA	Light	Avg. Cq	Cq Std. Dev.	Fold Reduction
Control (no virus)	+	-	undetermined		
Control (no virus)	+	+	undetermined		
Infectious MNV-1	-	-	23.38	0.02	
	-	+	23.65	0.04	1
	+	-	23.77	0.00	1
	+	+	23.95	0.06	1
Inactivated MNV-1 (replication 1)	+	-	23.33	0.18	1
	+	+	26.00	0.11	6 [b]
Control (no virus)	+	-	undetermined		
Control (no virus)	+	+	undetermined		
Infectious Norwalk Virus	-	-	33.75	0.01	
	-	+	33.73	0.30	1
	+	-	33.63	0.53	1
	+	+	33.85	0.10	1
Inactivated Norwalk virus	+	-	33.44	0.16	1
	+	+	34.45	0.15	2 [c]

[a] Each RT-qPCR reaction was done in duplicate, except for Inactivated Norwalk virus, where 3 separate assays using duplicate RT-qPCR assays were performed.

[b] There is a statistically significant difference between these values and all other values within the MNV-1 virus group ($P = <0.001$; power at alpha = 0.050 of 1.000).

[c] There is a statistically significant difference between this value and all other values within the Norwalk virus group ($P = <0.001$; power at alpha = 0.050 of 0.996), except with Infectious Norwalk virus with PMA and Light treatment.

Table 5. Cq values for PMA- RT-qPCR assay on infectious virus and viral RNA for different concentrations of PMA and different light exposure times [a]

Virus	PMA (µm)		Time of light exposure (min)		Avg. Cq	Fold Reduction
	348	696	3	5		
Control (no virus)		+		+	undetermined	
Infectious MNV-1		-		-	25.38	1
		+		-	26.01	2
		+		+	29.58	20 [b]
MNV-1 RNA	+	-	-	-	29.57	1
	+	-	+	-	>40	>1382 [b]
	+	-	-	+	>40	>1382 [b]
	-	+	-	-	30.11	2
	-	+	+	-	>40	>1382 [b]
	-	+	-	+	>40	>1382 [b]

[a] Each control and infectious MNV-1 RT-qPCR test was conducted in duplicate. Each MNV-1 RNA RT-qPCR test consisted of two separate experiments performed in duplicate.

[b] There is a statistically significant difference between these values and all other values ($P = <0.001$; power at alpha = 0.050 of 1.000).

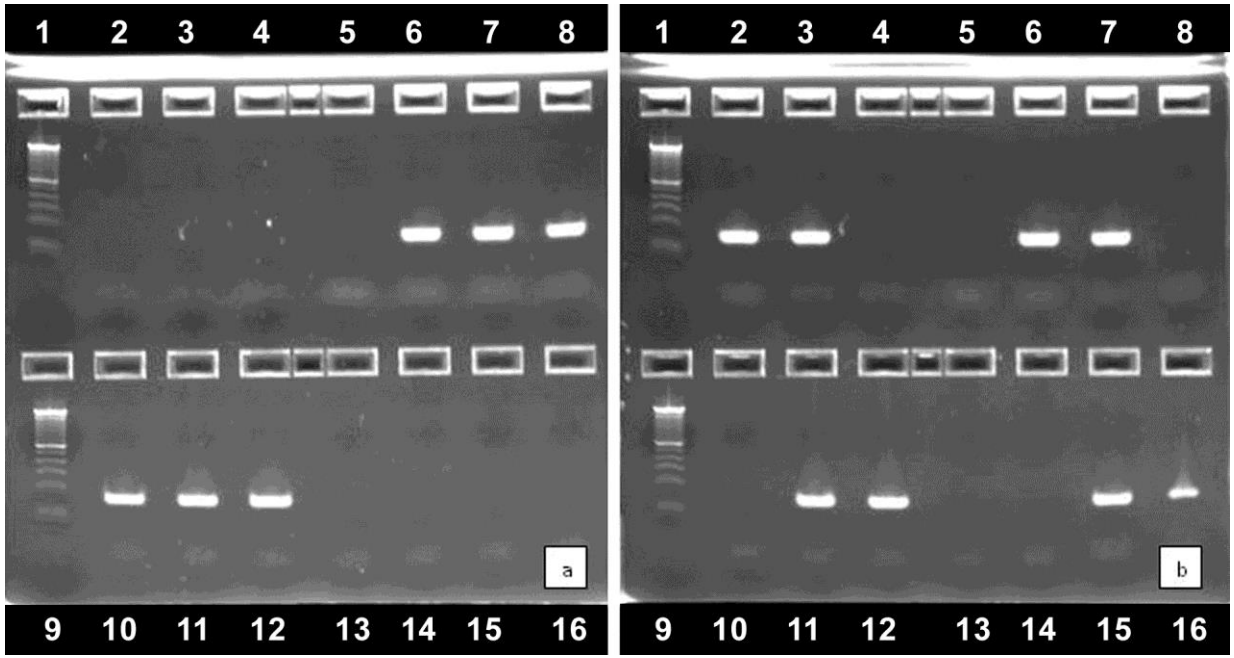

Figure 1: RT-PCR of Infectious and Heat-Inactivated Poliovirus 1

Poliovirus was treated with 348 µM PMA and exposed to light for 3 minutes. The virus-positive amplified product is 196 bp.

Gel a: Lane 1 and 9 = 100 bp ladder; Lane 2 and 3 = RT negative; Lane 4 and 5 = PCR negative; Lane 6 and 7 = infectious poliovirus treated with PMA but without the exposure to light; Lane 8 and 10 = infectious poliovirus treated with PMA and exposed to light; Lane 11 and 12 = infectious poliovirus treated with DMSO without exposure to light; Lane 13 and 14 = negative control (no virus) treated with PMA and light; Lane 15 and 16 = negative control (no virus) treated with PMA without light exposure.

Gel b: Lane 1 and 9 = 100 bp ladder; Lane 2, 3, 6, 7, 11, and 12 = non-infectious poliovirus treated with PMA but without the exposure to light; Lane 4, 5, 8, 10, 13, and 14 = non-infectious poliovirus treated with PMA and exposed to light; Lane 15 and 16 =infectious poliovirus without any treatment. Poliovirus was rendered non-infectious by heating to 72ºC for 5 seconds (Lanes 2 to 5), 6 seconds (Lanes 6 to 8, 10), or 7 seconds (Lanes 11 to 14).

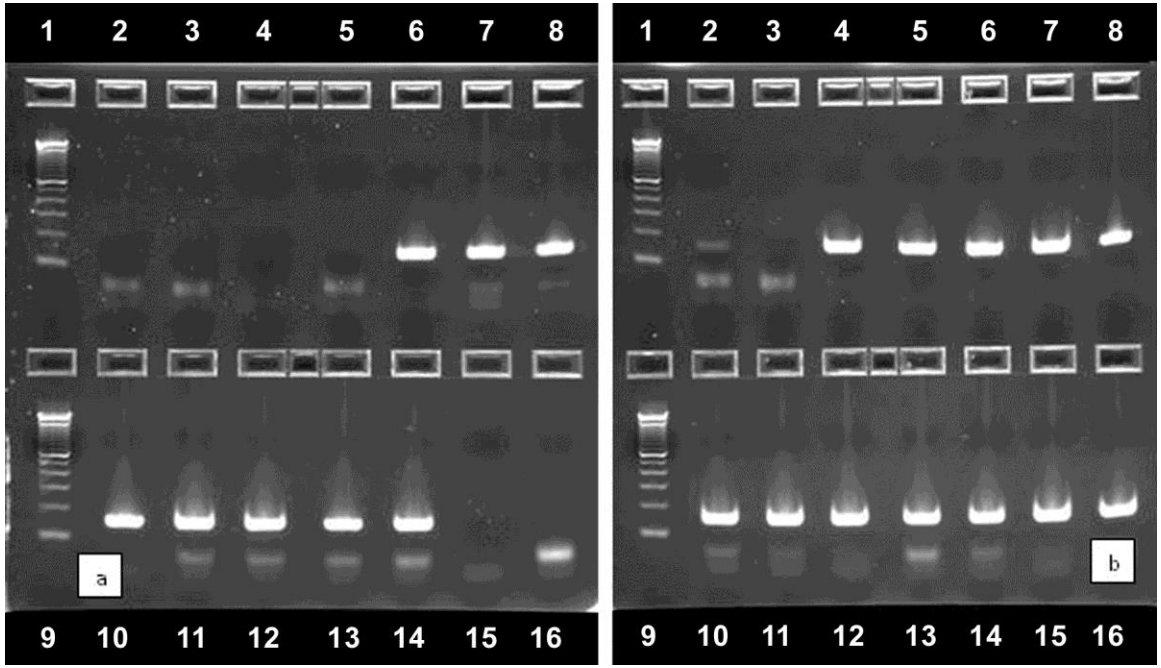

Figure 2: PMA RT-PCR of Infectious and Heat-Inactivated Murine Norovirus-1 (MNV-1)

MNV-1 was treated with 348 µM PMA and exposed to light from 3 minutes. The virus-positive amplified product is 159 bp.

Gel a: Lane 1 and 9 = 100 bp ladder; Lane 2 and 3 = RT negative; Lane 4 and 5 = PCR negative; Lane 6 and 7= infectious MNV-1 without any treatment; Lane 8 and 10 = infectious MNV-1 with only light exposure; Lane 11 and 12 = infectious MNV-1 treated with PMA and light; Lane 13 and 14= infectious MNV-1 treated with PMA without the exposure to light; Lane 15 and 16= negative control (no virus) treated with PMA and light.

Gel b: Lane 1 and 9 = 100 bp ladder; Lane 2 and 3 = negative control (no virus) treated with PMA without light; Lane 4, 5, 8, 10, 13, and 14 = non-infectious MNV-1 treated with PMA and light; Lane 6, 7, 11, 12, 15, and 16 = non-infectious MNV-1 treated with PMA without the exposure to light. MNV-1 was rendered non-infectious by heating to 72ºC for 5 minutes.

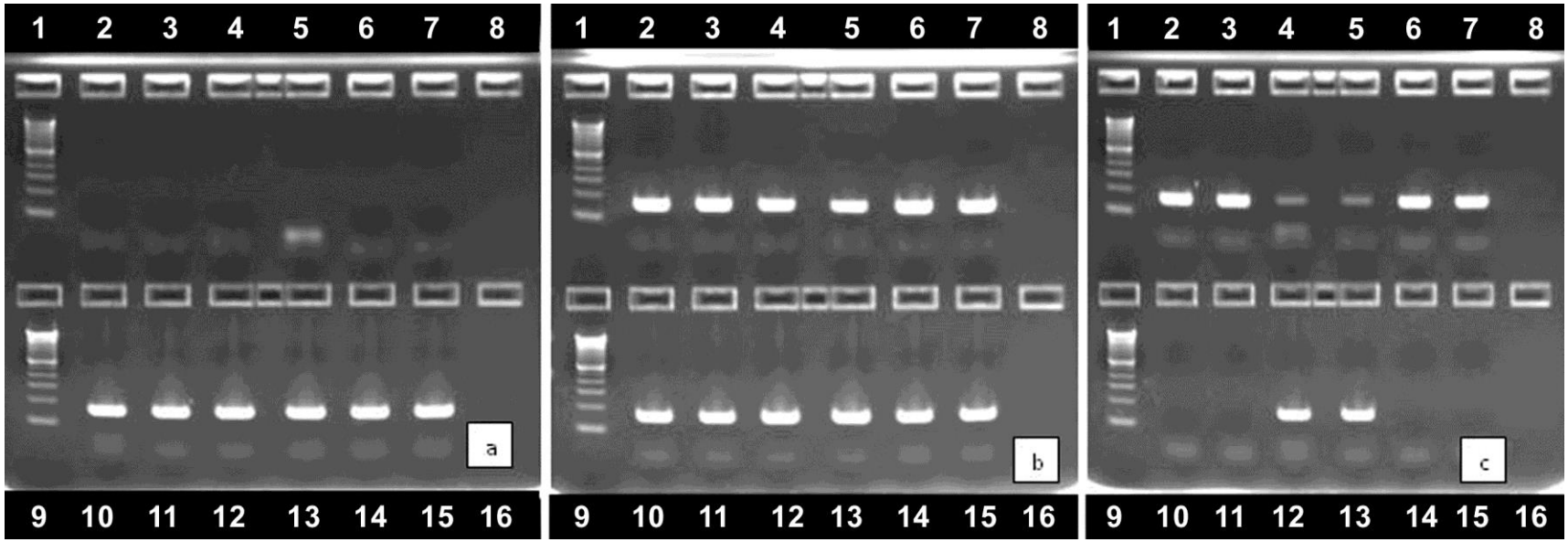

Figure 3: PMA RT-PCR of infectious and chlorine inactivated MNV-1

Gel a: Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= RT negative; Lane 4 and 5= PCR negative; Lane 6 and 7= negative control (no virus) treated with PMA without light exposure; Lane 8 = empty; Lane 10 and 11= infectious MNV-1; Lane 12 and 13= infectious MNV-1 treated with PMA without the exposure to light; Lane 14 and 15= infectious MNV-1 treated with PMA and light; Lane 16= empty. Gel b: (chlorine inactivated at CT value 0.06 mg/l) Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= non-infectious MNV-1 treated with PMA without exposure to light; Lane 4 and 5= non-infectious MNV-1 treated with PMA and light; Lane 6 and 7= non-infectious MNV-1 treated with PMA without exposure to light; Lane 8=empty; Lane 10 and 11= non-infectious MNV-1 treated with PMA and light; Lane 12 and 13= non-infectious MNV-1 treated with PMA without exposure to light; Lane 14 and 15= non-infectious MNV-1 treated with PMA and light; Lane 16=empty. Gel c: (chlorine inactivated at CT value 2.7 mg/l) Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= non-infectious MNV-1 treated with PMA without exposure to light; Lane 4 and 5= non-infectious MNV-1 treated with PMA and light; Lane 6 and 7= non-infectious MNV-1 treated with PMA without exposure to light; Lane 8=empty; Lane 10 and 11= non-infectious MNV-1 treated with PMA and light; Lane 12 and 13= non-infectious MNV-1 treated with PMA without exposure to light; Lane 14 and 15= non-infectious MNV-1 treated with PMA and light; Lane 16=empty.

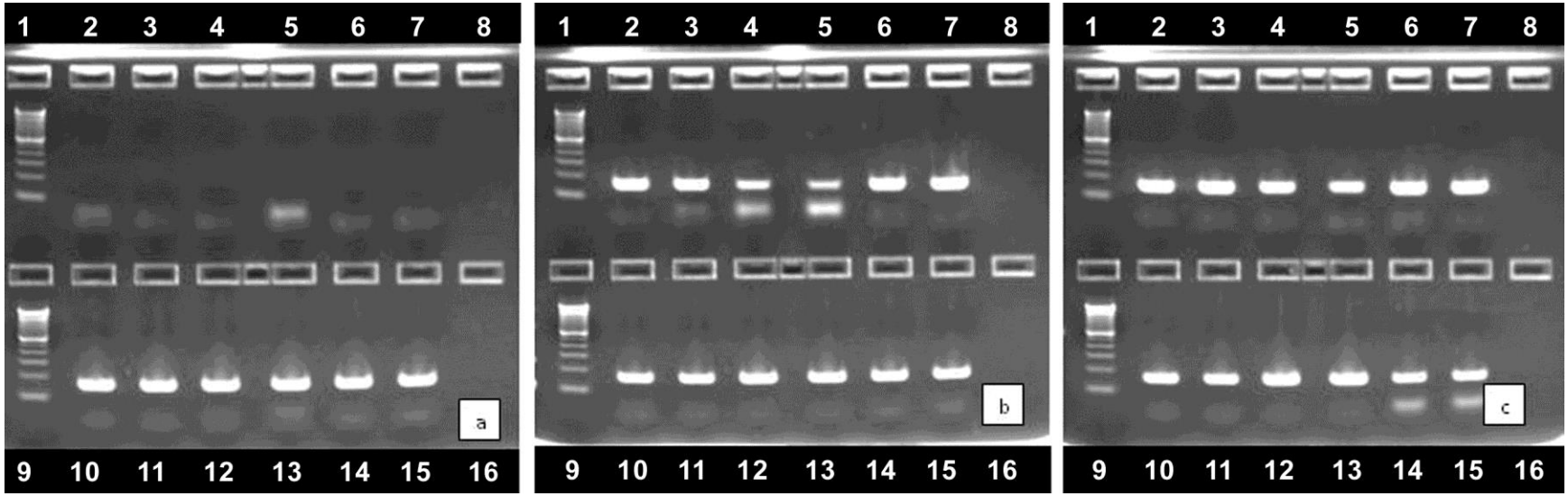

Figure 4: RT-PCR for infectious and UV disinfected MNV-1.

Gel a: Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= RT negative; Lane 4 and 5= PCR negative; Lane 6 and 7= negative control (no virus) treated with PMA without light exposure; Lane 8 = empty; Lane 10 and 11= infectious MNV-1; Lane 12 and 13= infectious MNV-1 treated with PMA without the exposure to light; Lane 14 and 15= infectious MNV-1 treated with PMA and light; Lane 16=empty. Gel b: (UV inactivated at 33 mJ/cm^2) Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= non-infectious MNV-1 treated with PMA without exposure to light; Lane 4 and 5= non-infectious MNV-1 treated with PMA and light; Lane 6 and 7= non-infectious MNV-1 treated with PMA without exposure to light; Lane 8=empty; Lane 10 and 11= non-infectious MNV-1 treated with PMA and light; Lane 12 and 13= non-infectious MNV-1 treated with PMA without exposure to light; Lane 14 and 15= non-infectious MNV-1 treated with PMA and light; Lane 16=empty. Gel c: (UV inactivated at 187 mJ/cm^2) Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= non-infectious MNV-1 treated with PMA without exposure to light; Lane 4 and 5= non-infectious MNV-1 treated with PMA and light; Lane 6 and 7= non-infectious MNV-1 treated with PMA without exposure to light; Lane 8=empty; Lane 10 and 11= non-infectious MNV-1 treated with PMA and light; Lane 12 and 13= non-infectious MNV-1 treated with PMA without exposure to light; Lane 14 and 15= non-infectious MNV-1 treated with PMA and light; Lane 16=empty.

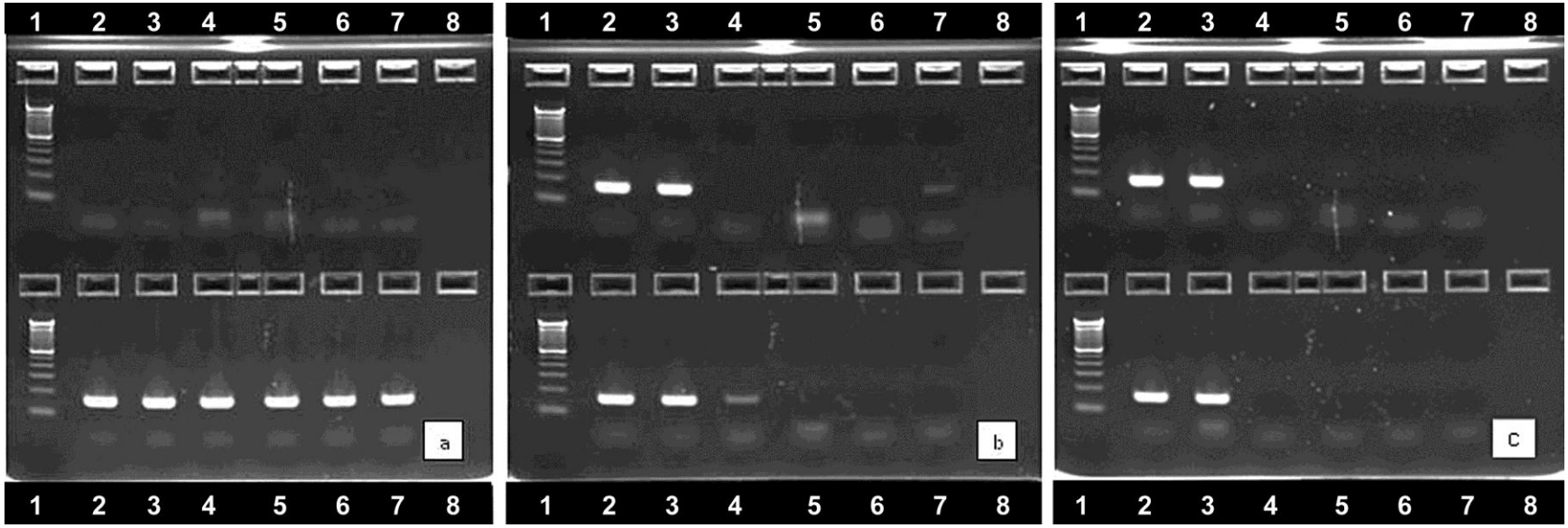

Figure 5: RT-PCR of naked MNV-1 RNA

Gel a: Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= RT negative; Lane 4 and 5= PCR negative; Lane 6 and 7= negative control (no virus) treated with PMA without light exposure; Lane 8 = empty; Lane 10 and 11= infectious MNV-1; Lane 12 and 13= infectious MNV-1 treated with PMA without the exposure to light; Lane 14 and 15= infectious MNV-1 treated with PMA and light; Lane 16=empty. Gel b (PMA concentration = 348 µm): Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= MNV-1 RNA treated with PMA without exposure to light; Lane 4 and 5= MNV-1 RNA treated with PMA and 3 min. of light exposure; Lane 6 and 7= MNV-1 RNA treated with PMA and 5 min. of light exposure; Lane 8= empty; Lane 10 and 11= MNV-1 RNA treated with PMA without exposure to light; Lane 12 and 13= MNV-1 RNA treated with PMA and 3 min. of light exposure; Lane 14 and 15= MNV-1 RNA treated with PMA and 5 min. of light exposure; Lane 16= empty. Gel c (PMA concentration = 696 µm): Lane 1 and 9 = 100 bp ladder; Lane 2 and 3= MNV-1 RNA treated with PMA without exposure to light; Lane 4 and 5= MNV-1 RNA treated with PMA and 3 min. of light exposure; Lane 6 and 7= MNV-1 RNA treated with PMA and 5 min. of light exposure; Lane 8= empty; Lane 10 and 11= MNV-1 RNA treated with PMA without exposure to light; Lane 12 and 13= MNV-1 RNA treated with PMA and 3 min. of light exposure; Lane 14 and 15= MNV-1 RNA treated with PMA and 5 min. of light exposure; Lane 16= empty.