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Use of ethidium monoazide and propidium monoazide to determine viral infectivity upon inactivation by heat, UV- exposure and chlorine

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ABSTRACT

Despite the great sensitivity of PCR in monitoring enteric viruses in an aquatic environment, PCR detects viral nucleic acids of both infectious and noninfectious viruses, limiting the conclusions regarding significance for public health. Ethidium monoazide (EMA) and propidium monoazide (PMA) are closely related membrane impermeant dyes that selectively penetrate cells with compromised membranes. Inside the cells, the dye can intercalate into nucleic acids and inhibit PCR amplification. To assess whether EMA and PMA pretreatment is a suitable approach to inhibit DNA amplification from noninfectious viruses upon heat treatment, UV exposure or chlorine treatment, viruses were measured by qPCR, EMA-qPCR, PMA-qPCR and cell culture titration. EMA/PMA-qPCR of UV- and heat-treated viruses did not correlate with the results of the cell culture assay. However, the data from EMA/PMA-qPCR of chlorine-inactivated viruses was consistent with the cell culture infectivity assay. Therefore, a dye treatment approach could be a rapid and inexpensive tool to screen the efficacy of chlorine disinfection, but it is not able to distinguish between infectious and noninfectious viruses inactivated via heat treatment or UV irradiation. Indeed, different viruses may have different trends and mechanisms of inactivation; thus, the assay must be evaluated for each virus separately.

Introduction

A wide range of analytical methods is available for virus detection in environmental samples, such as propagating the virus in animal tissue culture, polymerase chain reaction, and integrated cell culture PCR. Cell culture is the gold standard method to test virus infectivity. Some enteric viruses are easy to propagate, whereas others are difficult to propagate. However, no cell line is available for the propagation of human noroviruses by the conventional cell culture method. The assay is also not specific for particular viruses; therefore, the viral pathogen should be confirmed with other approaches, such as a molecular or immunological assay. In addition, virus propagation in cell culture is time consuming, labor-intensive and expensive; thus, it cannot be used as a routine and robust detection tool (Hamza et al., 2011b).

Molecular methods such as polymerase chain reaction (PCR) and real time PCR have the highest sensitivity and specificity to investigate virus contamination in water; therefore, they are the most commonly used methods in environmental virology (Mattison and Bidawid, 2009). PCR has the ability to detect naked nucleic acids and both infectious and non-infectious pathogens. Consequently, direct PCR does not allow for the discrimination between infectious and noninfectious viral particles. Although molecular methods have the highest degree of sensitivity and specificity, the co-concentration of PCR inhibitors from environmental water samples may represent a limitation for the use of PCR as a detection method (Girones et al., 2010).

Integrated cell culture—PCR (ICC-PCR) has the benefits of both cell culture and PCR. It attempts to compensate for several disadvantages in cell culture, such as time consumption and limited detection sensitivity. It has also been proposed as an alternative method for the detection of waterborne enteric viruses in environmental samples. A combination of cell culture with PCR has permitted the detection of infectious viruses that grow slowly or fail to produce cytopathic effects. ICC-PCR relies on an initial biological amplification of the viral nucleic acids, followed by real time (RT-) PCR amplifications. Using ICC-PCR, the presence of infectious enteroviruses could be confirmed as early as 1 day post-inoculation in comparison to 3 days or more by the traditional cell culture infectivity assay (Murrin and Slade, 1997; Reynolds et al., 1996). ICC-PCR/ICC-qPCR has been used for the detection of a wide variety of infectious human viruses, such as enteroviruses,

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adenoviruses, rotaviruses, hepatitis A virus, astroviruses and reoviruses, in aquatic environments (Abad et al., 1997; Balkin and Margolin, 2010; Ballester et al., 2005; Blackmer et al., 2000; Chapron et al., 2000; Grimm et al., 2004; Hamza et al., 2011a; Jiang and Chu, 2004; Lee et al., 2004; Rigotto et al., 2010; Shieh et al., 2008; Spinner and Di Giovanni, 2001). Although ICC-PCR detects virus infectivity faster than cell culture alone, this approach is still labor-intensive, and it requires at least 2 days before results can be obtained. In addition, it does not currently detect the infectious human norovirus group.

The enzymatic treatment prior to the extraction of viral nucleic acids was efficient for viruses that were inactivated by heat at 72 °C, chlorine, or UV exposure but not for viruses that were inactivated with a long exposure at 37 °C at which viral capsids still protect the viral nucleic acid (Nuanualsuwan and Cliver, 2003). In contrast, heat inactivation using enzymatic treatment has been reported to have a much stronger detrimental effect on virus infectivity than on the integrity of the viral genome, and no correlation was found between the detection of murine norovirus 1 RNA by real-time reverse transcription-PCR and the infectivity by plaque assay before and after heat (80 °C) exposure (Baert et al., 2008). Thus, protease and RNase treatment may not be applicable to all types of RNA viruses.

The presence of an intact membrane is one feature used to distinguish between living and dead cells. Ethidium monoazide (EMA) and propidium monoazide (PMA) are closely related nucleic acid-intercalating dyes with a photo-inducible azide group that covalently cross-links to the nucleic acids upon light exposure (Nocker et al., 2006). The dye can only enter cells with compromised cell walls and cell membranes (Rudi et al., 2005). The modified nucleic acid structure leads to a strong signal reduction in subsequent real-time PCR as a result of PCR inhibition. EMA and PMA have been proposed for selective detection of viable bacteria (Bae and Wuertz, 2009; Chang et al., 2010; Nogva et al., 2003; Pan and Breidt, 2007; Rudi et al., 2005; Wang et al., 2009), protozoa (Bertrand et al., 2009; Brescia et al., 2009; Fittipaldi et al., 2011; Sauch et al., 1991), nematode eggs (Christoforou et al., 2014), and fungi (Andorra et al., 2010; Vesper et al., 2008). However, few studies have examined the possibility of DNA/RNA-interacting dyes as a tool to distinguish between infectious and inactivated viruses after thermal and chlorine treatment (Coudray-Meunier et al., 2013; Graiver et al., 2010; Kim et al., 2011; Parshionikar et al., 2010).

Less concordance exists between published reports on this method due to the use of different virus types and different systems for light activation of EMA and PMA. In this context, the goal of the present study was to evaluate the applicability of both EMA-qPCR and PMA-qPCR for the discrimination between infectious and noninfectious viruses, such as adenovirus, poliovirus, murine norovirus, rotavirus and φX174, upon heat, UV or chlorine treatment.

Material and methods

Virus stock and culture

Human adenovirus type 5 (HAdV5) (kindly provided by the molecular virology lab at Ruhr University Bochum) was grown in T293 cells. Poliovirus type 1 Sabin (PV1) (kindly provided by the Robert Koch Institute, Germany) was grown in BGM cells. The cells were propagated in Dulbecco MEM (DMEM; Sigma) supplemented with 10% FBS and 1% penicillin-streptomycin. Rotavirus (RoV) (kindly provided by the Robert Koch Institute, Germany) was propagated in MA104 cells (purchased from ECACC). The cells were grown in Eagle minimum essential medium (MEM; Sigma) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% nonessential amino acids, and 1% l-glutamine. Murine norovirus (MNV) (provided by the Friedrich Loeffler Institute, Germany) was propagated in RAW 264.7 cells (ECACC). RAW 264.7 cells were cultured in 1× RPMI 1640 medium supplemented with 10% FBS. The bacteriophage ΦX174 was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and was propagated in *Escherichia coli* (ATCC 13706).

Virus titration

The end-point dilution assay was used to measure virus titer on virus-susceptible cell lines as described above. The virus concentration was calculated following the method of Reed and Muench (1938). The cells were seeded into 48-well tissue culture plates at a density of 5×10^4 cells per well. The cells were then allowed to adhere for 48 h at 37 °C in the presence of 5% CO_2. The cell culture medium was decanted, and the cells were washed with PBS and infected with 50 μl of each serially diluted inoculum in PBS for 60 – 90 min at 37 °C in the presence of 5% CO_2. Afterward, the inocula were aspirated and replaced with 200 μl of maintenance medium and incubated for 3 – 5 days at 37 °C in the presence of 5% CO_2. The infected cells were monitored for the development of CPE during the incubation period, and the virus concentration was expressed as TCID50/ml.

Quantification of coliphage φx174

Coliphage φx174was quantified by using the double agar layer plaque test according to the standard method of the international organization for standardization, ISO 10705-2 (ISO, 2002). *E. coli* DSM 13127 grown on modified Scholten's broth (MSB) was used as a host strain for the quantification of φx174. One milliliter of an exponentially growing host strain, 100 μl of treated or non-treated coliphage sample and 2.5 ml of molten agar (Scholten's modified semi-solid agar) were mixed and then poured onto previously prepared modified Scholten's agar plates. Plaques were counted within 3 – 5 h of incubation at 37 °C and calculated as PFU/ml.

Thermal inactivation of viruses

Viruses were suspended in PBS and filtrated using a 0.22-μm cellulose acetate syringe filter (Chromafil, Macherey Nagel; Germany). Viruses were inactivated thermally in phosphate-buffered saline (PBS) pH 7.0. Two-milliliter aliquots were incubated for 10 min in water baths set at 45 °C, 55 °C and 65 °C to achieve different degrees of viral inactivation. One aliquot of virus suspension was kept on ice during heat treatment and used as a control. After thermal inactivation, samples were kept on ice and further subjected to EMA or PMA treatment followed by EMA/PMA-qPCR and an infectivity assay.

UV-light treatment

Viruses were suspended in PBS and filtrated using a 0.22-μm cellulose acetate syringe filter (Chromafil, Macherey Nagel; Germany). Aliquots of virus suspension were pipetted into 6-well tissue culture plates, and the lid was removed for direct UV light exposure. The plates were placed 20 cm below a low pressure 25 W germicidal UV lamp (TUV25WG13 UV-C, Philips) in a UV cabinet (UVC/T-AR; Biosan). The bulb emitted monochromatic and germicidal light at a peak wavelength of 253.7 nm, and the UV intensity was 1.9 ± 0.47 mW/cm^2 as measured by Ocean Optics QE65000 spectrometer. The UV exposures of 30 s, 60 s, and 120 s