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Viral persistence in surface and drinking water: Suitability of PCR pre-treatment with intercalating dyes

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ABSTRACT

After many outbreaks of enteric virus associated with consumption of drinking water, the study of enteric viruses in water has increased significantly in recent years. In order to better understand the dynamics of enteric viruses in environmental water and the associated viral risk, it is necessary to estimate viral persistence in different conditions. In this study, two representative models of human enteric viruses, adenovirus 41 (AdV 41) and coxsackievirus B2 (CV-B2), were used to evaluate the persistence of enteric viruses in environmental water. The persistence of infectious particles, encapsidated genomes and free nucleic acids of AdV 41 and CV-B2 was evaluated in drinking water and surface water at different temperatures (4 °C, 20 °C and 37 °C). The infectivity of AdV 41 and CV-B2 persisted for at least 25 days, whatever the water temperature, and for more than 70 days at 4 °C and 20 °C, in both drinking and surface water. Encapsidated genomes persisted beyond 70 days, whatever the water temperature. Free nucleic acids (i.e. without capsid) also were able to persist for at least 16 days in drinking and surface water. The usefulness of a detection method based on an intercalating dye pre-treatment, which specifically targets preserved particles, was investigated for the discrimination of free and encapsidated genomes and it was compared to virus infectivity. Further, the resistance of AdV 41 and CV-B2 against two major disinfection treatments applied in drinking water plants (UV and chlorination) was evaluated. Even after the application of UV rays and chlorine at high doses (400 mJ/cm^2 and 10 mg.min/L, respectively), viral genomes were still detected with molecular biology methods. Although the intercalating dye pre-treatment had little use for the detection of the effects of UV treatment, it was useful in the case of treatment by chlorination and less than 1 $\log_{10}$ difference in the results was found as compared to the infectivity measurements. Finally, for the first time, the suitability of intercalating dye pre-treatment for the estimation of the quality of the water produced by treatment plants was demonstrated using samples from four drinking-water plants and two rivers. Although 55% (27/49) of drinking water samples were positive for enteric viruses using molecular detection, none of the samples were positive when the intercalating dye pre-treatment method was used. This could indicate that the viruses that were detected are not infectious.

1. Introduction

Despite the release of large quantities of human pathogens into rivers by wastewater treatment plant (WWTP) effluents (Prevost et al., 2015; Zhou et al., 2015), surface water is used widely for the production of drinking-water (Grondahl-Rosado et al., 2014), for recreational activities (Allmann et al., 2013) and for agricultural irrigation (Cheong et al., 2009). Among the waterborne pathogens, human enteric viruses are a major cause of acute gastroenteritis worldwide. They also may cause diverse additional disorders such as hepatitis, conjunctivitis or neurological lesions. In addition, they confer a high risk of morbidity and mortality in vulnerable populations such as young children, immunocompromised patients and elderly individuals (Gallimore et al., 2004). The number of studies focusing on enteric viruses in drinking water and foods has increased recently, which is due partly to several outbreaks of gastroenteritis that have been associated with food or water consumption and to the improvements in detection methods (Iritani et al., 2014; Loury et al., 2015; Morillo et al., 2012). Currently,

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molecular methods such as quantitative polymerase chain reaction (qPCR) or reverse transcriptase-qPCR (RT-qPCR) are used widely for viral detection and quantification. Nevertheless, the gold standard remains the titration of virus on permissive cells which provides information about virus infectivity. The lack of cell lines for some enteric viruses, and more particularly for norovirus, explains the success of the molecular biology methods (Neesanant et al., 2013). These methods present the advantage of being highly sensitive and they allow the detection of low virus levels. This is important since small amounts of human enteric viruses may be sufficient to provoke a rapid infection, given that they have a low infectious dose (between 10 and 100 viral particles). However, the detection of viral nucleic acids does not imply infectious risk. Although several studies have reported positive viral analyses of food and drinking water, no discrimination was made between infectious and non-infectious viral particles (Heerden et al., 2005; Kokkinos et al., 2012). In order to assess viral integrity, some authors recommend that samples be treated with intercalating dyes such as ethidium monoazide (EMA) or propidium monoazide (PMA) before nucleic acid are extracted and amplified (Coudray-Meunier et al., 2013; Karim et al., 2015). Two hypotheses underly the use of intercalating dyes:i) a virus with a damaged capsid is not infectious, ii) intercalating dyes can reach and bind to genomes to block specifically the amplification of defective particles. However, some studies have suggested that the utilization of intercalating dyes is not always relevant to discriminate infectious from non-infectious viral particles, and that such discrimination is dependent on the mechanisms of virus inactivation (Kim et al., 2011; Leifels et al., 2015). Indeed, heat exposure tends to destroy the viral capsid, chlorine or ozone treatments may damage both viral capsid and nucleic acids depending upon the exposure doses, whereas UV rays tend to target mainly the nucleic acids. Currently, the experiments using intercalating dye pre-treatment to assess viral integrity have been conducted only under laboratory conditions with defined quantities of purified viral particles spiked into the water samples. No experiments have been conducted using environmental samples that contain indigenous enteric viruses.

The main goal of this study was to evaluate the persistence of enteric viruses in water samples and to determine the suitability of using pre-treatment with intercalating dyes to detect viruses in water samples. The efficiency of EMA and PMA to prevent genome amplification was evaluated using different types of viral nucleic acids: double strand DNA or RNA, positive and negative single strand RNA. Then, the persistence of human enteric viruses (using adenovirus 41 (AdV 41) and coxsackievirus B2 (CV-B2) as models) was estimated in samples of surface water and drinking water exposed to different temperatures. Also, the impact on viral resistance of two major disinfection treatments (chlorine and UV irradiation) generally used in drinking water plants was estimated. Finally, the relevance of intercalating dye pre-treatment in the monitoring of viral contamination in drinking water and river water was evaluated with an extensive sampling campaign.

2. Materials and methods

2.1. Viral stock preparation

AdV 41 and CV-B2 strains were cultivated on monolayer cultures of 293A cells and Buffalo green monkey kidney (BGMK) cells, respectively, at 37 °C with 5% CO_2. The 293A cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 5% foetal bovine serum (FBS), glutamine and non-essential amino acids. The BGMK cells were grown in Dulbecco's Modified Eagle's Medium high glucose supplemented with 5% foetal bovine serum (FBS) and non-essential amino acids. To harvest viruses, the cells were lysed by three consecutive freeze–thaw cycles and the supernatant containing the viral particles was clarified by centrifugation at 1500 ×*g* for 10 min. AdV 41 and CV-B2 suspensions were purified on a sucrose gradient and then quantified by viral titration and by (RT)-qPCR. Purified viruses were stored at −80 °C until use. Rotavirus A (RV-A) was extracted from stool samples and Influenza virus A/California/07/09 was kindly provided by the French Reference National Centre for Influenza virus.

These four viruses were used to obtain different types of free nucleic acids (double-strand DNA or RNA, positive and negative single-strand RNA). AdV 41 and CV-B2 strains also were used to evaluate the impact of different conditions (temperature, type of water and disinfection treatment) on viral persistence.

2.2. Ethidium and propidium monoazide treatments

2.5 mM solutions of the intercalating dyes EMA and PMA were prepared in molecular grade water and aliquots were stored at −20 °C. Each aliquot underwent only one freeze–thaw cycle. Preliminary studies were leaded to define the optimal concentration of the dyes concentration which allowed the blocking of a maximum of the free genome with a minimum in inhibition of PCR (data not shown). Based on these results, each pre-treatment with EMA or PMA was performed with 0.02 μmole of dye for 100 μL of sample. Then, samples were mixed and incubated on ice for 30 min in the dark after which photo-activation was performed with a PhaST Blue system (IUL, Barcelona, Spain) for 15 min. After the intercalating dye pre-treatment of the water samples, the nucleic acids were extracted.

2.3. Extraction of viral nucleic acid

All viral nucleic acids were extracted, according to the manufacturer's instructions, with a MagNA Pure Compact instrument and MagNa Pure Compact Nucleic Acid Isolation Kit I – Large Volume (Roche Applied Science, Bâle, Switzerland), which allowed the processing of samples up to 1 mL. The extracted nucleic acids were immediately purified with OneStep™ PCR Inhibitor Removal Kit (Zymo Research Corporation, Irvine, CA) according to the manufacturer's instructions, then analyzed and any sample remaining was stored at −80 °C.

2.4. Real-time PCR assay conditions

Each independent reaction was carried out with 5 μL of nucleic acids, using specific primers and probes for each virus. All the primers that were used were described previously by Prevost & al for adenovirus (AdV), astrovirus (AstV), norovirus of genogroups I and II (NoV GI and NoV GII) and rotavirus A (RV-A) (Prevost et al., 2015), by Wurtzer & al for enterovirus (EV) (Wurtzer et al., 2014), by Nielsen & al for aichivirus (AichiV), cosavirus (CosaV) and salivirus (SaliV) (Nielsen et al., 2013), in the WHO guidelines for Influenza virus (W.H.O.G, 2011) and by Oka & al for sapovirus (SapoV) (Oka et al., 2006). Amplifications were performed with TaqMan® Fast Virus 1-Step Master Mix (Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations. All reactions were performed with a ViiA™ 7 real-time PCR system (Life Technologies, Carlsbad, CA). The thermal cycling profile was one step of reverse transcription at 50 °C for 5 min (this step not needed for adenovirus amplification), one step of initial denaturation at 95 °C for 20s, 45 cycles of 5s denaturation at 95 °C and 40s annealing/extension at 60 °C. Fluorescence was measured at the end of the annealing/extension step on the FAM, HEX and DFO channels. Each amplification run included a no template control and a positive amplification control. The results reported for each