



Quantification of infectious *Human mastadenovirus* in environmental matrices using PMAxx-qPCR

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Abstract

Molecular methodologies providing data on viral concentration and infectivity have been successfully used in environmental virology, supporting quantitative risk assessment studies. The present study aimed to assess *human mastadenovirus* (HAdV) intact particles using a derivative of propidium monoazide associated with qPCR (PMAxx-qPCR) in aquatic matrices. Initially, different concentrations of PMAxx were evaluated to establish an optimal protocol for treating different naturally contaminated matrices, using 10 min incubation in the dark at 200 rpm at room temperature and 15 min of photoactivation in the PMA-Lite™ LED photolysis device. There was no significant reduction in the quantification of infectious HAdV with increasing concentration of PMAxx used (20 μM, 50 μM, and 100 μM), except for sewage samples. In this matrix, a reduction of 5.01 log of genomic copies (GC)/L was observed from the concentration of 50 μM and revealed 100% HAdV particles with damaged capsids. On the other hand, the mean reduction of 0.51 log in stool samples using the same concentration mentioned above demonstrated 83% of damaged particles eliminated in the stool. Following, 50 μM PMAxx-qPCR protocol revealed a log reduction of 0.91, 0.67, and 1.05 in other samples of raw sewage, brackish, and seawater where HAdV concentration reached 1.47×10^4 , 6.81×10^2 , and 2.33×10^2 GC/L, respectively. Fifty micrometers of PMAxx protocol helped screen intact viruses from different matrices, including sea and brackish water.

Keywords Propidium monoazide · PMAxx-qPCR · HAdV · Raw sewage · Brackish water · Seawater

Introduction

Diagnostic methods for viral detection and quantification have been investigated over the years, stimulating the development of innovative tools and technologies in virology [1]. Evaluation of infectious and non-infectious viruses is essential in environmental virology [2, 3] since the quantification of infectious viruses is a determining factor for calculating the quantitative risk assessment of exposure of susceptible individuals to contaminated environmental matrices [4, 5].

Viral quantification methodologies to estimate values of viral particles as the viral plaque assay using plaque-forming

units (PFU) per ml, median tissue culture infectious dose (TCID₅₀), or quantitative polymerase chain reaction (qPCR) have been used. The last one cannot distinguish between infectious and non-infectious viruses [6–10].

Cell culture has been the standard gold method in virology to assess viral infectivity; however, integrated cell culture quantitative PCR (ICC-qPCR) protocols are commonly used when dealing with environmental matrices and enteric viruses considered fastidious [11]. The ICC-qPCR method is laborious and costly but widely used for *human mastadenovirus* (HAdV) due to its ability to replicate in cell culture. However, cultivation in different cell types to increase the chances of isolation of serotypes is still needed [12]. HAdV has been described as a potential indicator of fecal contamination in environmental matrices mainly due to their high concentration and prevalence in sewage samples, resistance to environmental conditions, and absence of seasonality [13, 14].

In this scenario, a dye propidium monoazide (PMA) or its derivative (PMAxx) combined with qPCR (PMAxx-qPCR) has been pointed to as a faster, specific, and sensitive

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alternative approach to assessing capsid-integrity of enteric viruses in the environment, thus quantifying potentially infectious viral particles [15–21]. PMA is a nucleic acid intercalating dye with a photoinducible azide group that covalently binds to RNA/DNA from virion with damaged capsid, thus inhibiting genomic target amplification during qPCR [22, 23]. Factors such as viral species, dye concentration, incubation conditions, and light source for dye photoactivation influence PMAxx-qPCR efficacy and applicability [24]. In this context, this study aimed to assess PMAxx-qPCR for quantifying HAdV intact particles from different environmental matrices such as raw sewage, brackish water, and seawater.

Materials and methods

Experimental design and HAdV-positive controls

To quantify HAdV protocols using different concentrations of PMAxx (20, 50, and 100 μM) were first evaluated in three independent experiments using clinic and environmental HAdV-positive samples with known quantification. Stool samples were obtained from the Rotavirus

Reference Service/Laboratory of Comparative and Environmental Virology (LVCA), Oswaldo Cruz Institute (IOC/Fiocruz) in Rio de Janeiro, Brazil, with approval of the Research Ethics Committee of the Oswaldo Cruz Foundation (CAAE number: 94144918.3.0000.5248). For analysis, stool suspensions (10% w/v) were prepared in Tris/calcium buffer (0.1 M Tris/HCl; 1.5 mM CaCl₂; pH 7.2). Raw sewage, brackish, and seawater samples previously concentrated by skimmed milk flocculation method were obtained at LVCA. All clinical and environmental samples were obtained and analysed and kept at $-70\text{ }^{\circ}\text{C}$ until used in this study.

Sampling and viral concentration method

Posteriorly, another 30 environmental samples were collected, concentrated, and analysed to quantify the presence of HAdV intact capsids using the PMAxx-qPCR established protocol. These environmental samples were collected in the Barra da Tijuca, a coastal Metropolitan Region of Rio de Janeiro City, Brazil, from 2016 to 2020 (Fig. 1). Raw sewage, brackish, and seawater samples, were concentrated by skimmed milk flocculation method described for wastewater and recreational water samples [25, 26].

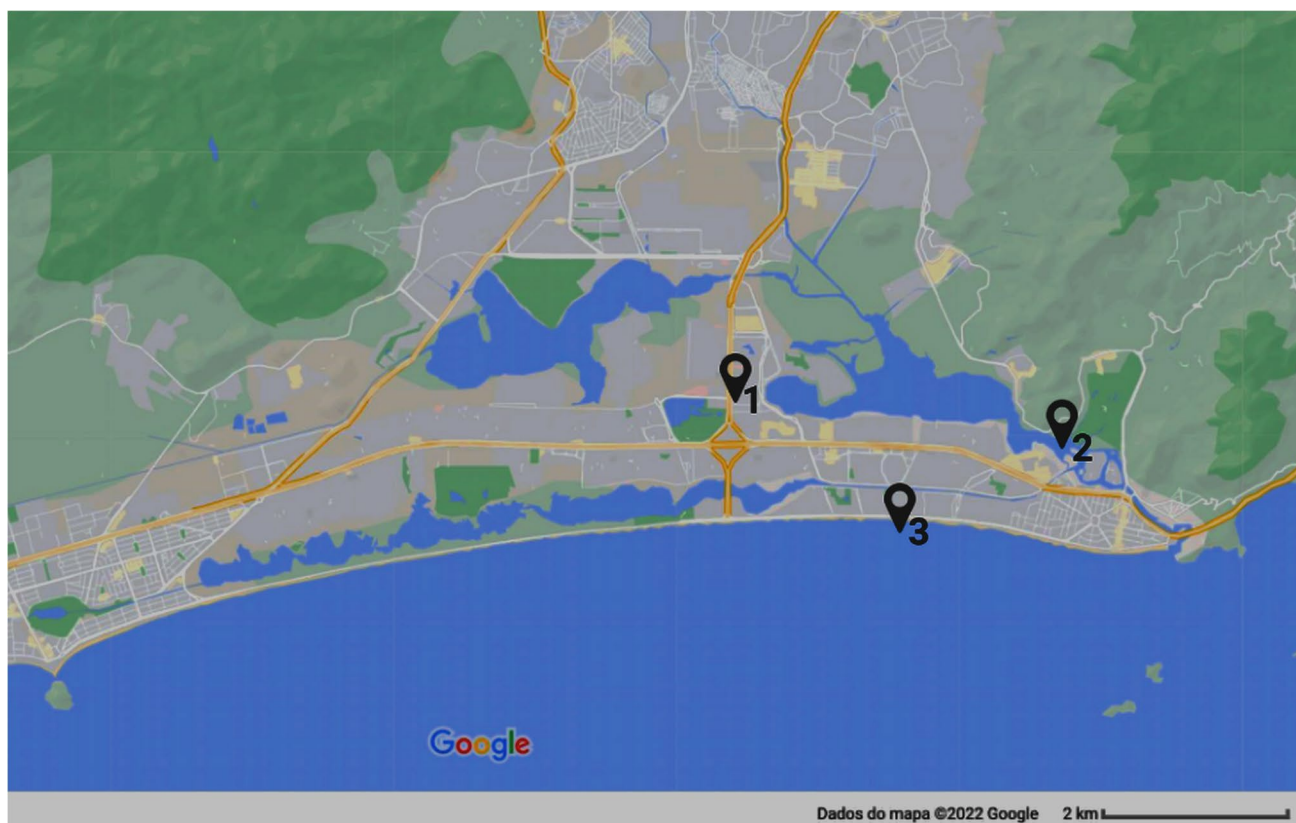


Fig. 1 Location of collection points indicating the environmental matrices studied: raw sewage (1) brackish water (2) and seawater (3)

PMAXx treatment

Stock solutions of PMAXx (Biotium Inc., Hayward, CA) at a concentration of 20 mM were diluted in ultrapure water to a working concentration of 500 μ M and stored at -20°C . Different samples from each matrix were aliquoted into four fractions of 140 μ L, three of them treated with PMAXx at concentrations of 20, 50, and 100 μ M and one without treatment. Based on a previous study using HAdV PMAXx qPCR, we designed a protocol incubating all treated and untreated samples at room temperature for 10 min at 200 rpm in the dark, followed by photoactivation for 15 min using the blue light of the PMA-Lite™ LED Photolysis Device (Biotium) [19, 22, 24, 27]. During PMAXx treatment, the samples were protected from light. The optimal PMAXx concentration was measured by comparing the results of the treated and untreated fractions of each matrix by reducing HAdV DNA amplification in the qPCR in genomic copies (GC)/L.

For interpretation of potentially infectious HAdV by matrix, PMAXx reduction log value was defined by the formula below:

$$\text{PMAXx log reduction value} = \log \text{qPCR untreated} - \log \text{qPCR treated}$$

The percentage of HAdV with damaged capsids was calculated using the following formula accordingly [16]:

$$\% \text{HAdV with damaged capsids} = 100 - \left[\frac{\log \text{qPCR treated}}{\log \text{qPCR untreated}} \times 100 \right]$$

Results of quantification in GC/L of samples treated with PMAXx and untreated samples were compared, numerically estimating the intact and potentially infectious HAdV per matrix. The same incubation and photoactivation conditions mentioned above were applied with the 30 samples tested for the PMAXx protocol to establish after choosing the appropriate concentration.

DNA extraction and quantification

According to the manufacturer's instructions, nucleic acids were manually extracted after the PMAXx treatment using the commercial QIAmp viral RNA mini kit (Qiagen™, Valencia, USA). The DNA extraction assay included negative control composed of DNase/RNase free water and positive controls of each matrix.

For detection and quantification of HAdV by qPCR, 25 μ L reaction mixtures consisting of 12.5 μ L of TaqMan Universal Master Mix II with UNG (Applied Biosystems™, Foster City, CA), 1 μ L of each primer (22.5 μ M), 0.5 μ L of probe and 10 μ L of nucleic acid. Specific HAdV primers and probes were used according to Hernroth et al. [28]. The target genomic fragments were amplified using ABI PRISM

7500 Real-Time TaqMan System. The qPCR conditions following activation of the uracil N-glycosylase (2 min, 50°C) and activation of the AmpliTaq Gold for 10 min at 95°C , 40 cycles (15 s at 95°C and 1 min at 60°C) and submitted to an infinite process at 4°C . Negative and positive controls from nucleic acid assay and NTC were included in all procedures. All undiluted and 1:10 to 1:100 diluted nucleic acid samples were tested in triplicate to avoid inference with possible qPCR inhibitors. Standard curves were designed using gBlock gene fragments (Integrated DNA Technologies™, Coralville, Iowa, USA) with HAdV sequence fragments. Nucleic acid was quantified by Qubit Fluorometer (Thermo Fisher Scientific™), and ten-fold dilutions were prepared and stored at -70°C until use. Serial dilutions (10^0 to 10^7 GC/reaction) presented the values of the slope, squared regression coefficient, and reaction efficiencies of -3.332 , 0.997 , and 99.7% , respectively. Values of $Ct \leq 38$ (presenting the characteristic sigmoidal curve) were considered positive.

Statistical analysis

Results were plotted using GraphPad Prism software version 8.0.1 (GraphPad Software, La Jolla, CA). A log transformation normalized data related to the number of GC/L. Differences between HAdV quantification in PMAXx-treated and untreated in different matrices were analysed using a parametric one-way ANOVA model, followed by Tukey's multiple comparison tests ($p < 0.05$).

Results

Figure 2 shows the quantification by GC/L of HAdV obtained by different concentrations of PMAXx used according to the matrices evaluated. Comparing PMAXx concentrations, no significant reduction when 20, 50, or 100 μ M ($P > 0.986$) were used as treatment independent of the matrices studied was observed, except for sewage samples. A mean value reduction of 5.01 logs GC/L was observed in this matrix when treated with 50 μ M of PMAXx.

HAdV viral particles' mean percentage revealed 99% and 95% of defective capsids in raw sewage and seawater, respectively (Table 1). In contrast, stool samples showed the lowest percentage (78%) of damaged capsid virus, followed by brackish water samples with 81%. The absence of intact HAdV particles in raw sewage was observed after the PMAXx treatment of 50 or 100 μ M.

Following, 30 environmental samples, 10 from each matrix, were processed using [50 μ M] PMAXx established protocol. A reduction of 0.91, 0.67, and 1.05 for raw sewage, brackish, and seawater, was observed with a mean

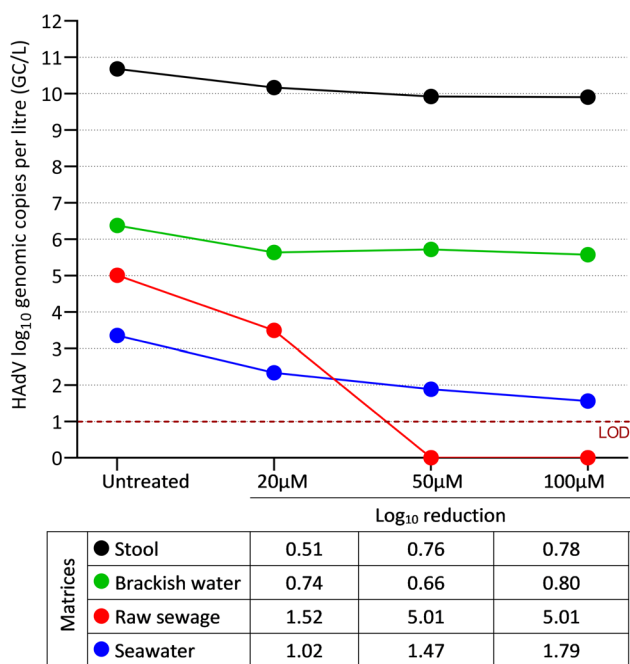


Fig. 2 Average reduction in the logarithmic basis of genomic copy values per litre of *Human mastadenoviruses* (HAdV) after treatment with different concentrations of PMAxx compared to untreated in the qPCR evaluated matrices ($n = 3$ per matrix). LOD: limit of detection

Table 1 Percentage of *Human mastadenoviruses* (HAdV) with damaged capsids in different matrices after PMAxx treatment

PMAxx concentration (μM)	Percentage of damage HAdV particle			
	Stool	Brackish water	Raw sewage	Seawater
20	69	82	97	90
50	83	78	100	97
100	83	84	100	98
Average value	78	81	99	95

concentration of 1.47×10^4 , 6.81×10^2 , and 2.33×10^2 HAdV infectious GC/L, respectively (Fig. 3). The average value percentage of HAdV viral particles revealed 88%, 79%, and 91% of defective capsids in those matrices, respectively.

Discussion

Treatment with PMA proved helpful in quantifying intact HAdV particles in all matrices evaluated, regardless of the concentration used. However, 50 μM of PMAxx presented an intermediate effect for inhibiting the signal amplification by qPCR compared to the other two concentrations, furthermore presented the best cost-benefit ratio with higher

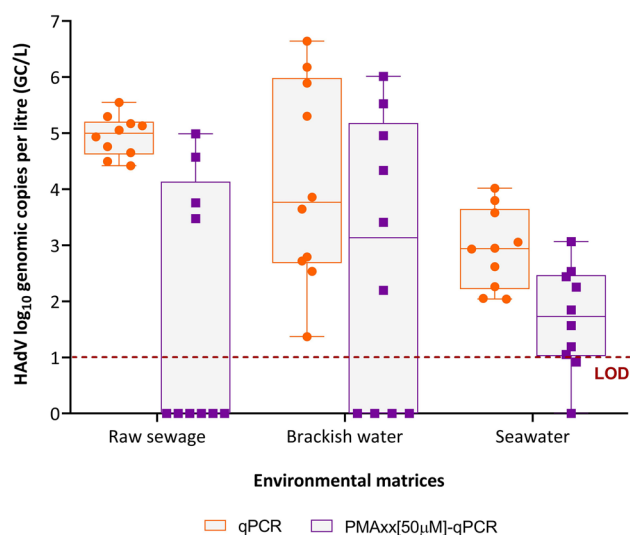


Fig. 3 Quantification of *Human mastadenoviruses* (HAdV) in environmental matrices analysed by qPCR and 50 μM PMAxx-qPCR ($n = 10$ per matrix). LOD, limit of detection

dye yield. According to recent systematic literature, many authors also used azo dyes at 50 μM in their experiments [29]. Until the completion of the present study, PMA and PMAxx were not applied to brackish and seawater matrices [29] and few studies have quantified intact HAdV particles using these dyes in environmental matrices [21, 30–32]. Only one study used PMAxx dye to analyse HAdV inoculated in phosphate-buffered saline (PBS) solution in thermal and free-chlorine disinfection processes [20]. The percentage of damaged capsid observed in this study was high, ranging from 69 to 100%, regardless of the concentration of PMAxx and matrix evaluated. Faeces and brackish samples showed the lowest amount of HAdV particles with damaged capsids. This quantification was confirmed when new brackish samples were evaluated, showing a variation of 157 to 1 million intact viruses, thus evidencing the high contamination of domestic sewage discarded in natura in this Lagoon used for recreational activities [33]. In seawater samples, despite the low concentration (11.3 to 1160), it was possible to detect intact HAdV. Although we cannot determine the origin of this contamination, the high concentration of those viruses in adjacent lagoons that exchange water with the sea is noteworthy. In this study, we did not assess the presence of marine currents or point and diffuse sources of faecal contamination, such as rainwater runoff and outfalls that may influence the increase or reduction of the spread and concentration of these infectious agents [34, 35]. So far, there is no study assessing HAdV PMAxx-qPCR in brackish or seawater.

The detection of HAdV intact particles with mean quantification of 10^3 log of HAdV GC/L in the sample treated with PMAxx, indicates the need for risk assessment studies for

the exposed population to these matrices. In addition, annual risk calculations for HAdV infection in recreational waters are up to 1/1000 for a single exposure [36]. Previously, our group evidenced viral contamination on other beaches on the coast of the city of Rio de Janeiro, showing concentrations as high as 10^4 log of GC/L [37], thus highlighting the need for further analyses in environmental samples. It is important to note that concentrations of infectious HAdV in environmental matrices are influenced by several factors capable of changing viral stability including the concentration method and the peculiarity of each matrix. The organic flocculation method based on skimmed milk has been described as a low-cost methodology, although it presents inhibition [38], which in this study was bypassed by the dilution of the samples. The number of polluting sources of raw sewage discharge; the geomorphology and hydrology of the collection site; the physicochemical elements (temperature, pH, oxidation-reduction potential, salinity, organic, and inorganic components, among others), the presence of microorganisms with virucidal or predation action; suspended solid particles (viral aggregation), and ultraviolet light irradiation should be considered [13, 39–42].

Our results using PMA_{xx} showed a reduction ranging from 0.91 to 5 log, unlike the result obtained in a previous study carried out for HAdV in sewage samples, where a logarithmic reduction of 0.74 was observed [22]. However, regarding the quantification of infectious HAdV, our results were similar to those found in the same study (2.69×10^4 TCID₅₀/L and 2.27×10^5 GC/L). It is important to emphasize that although PMA_{xx}-qPCR provides more reliable data than qPCR, it may overestimate the real numbers of infectious viruses, as described in studies of viral inactivation after heat, chlorine and UV treatments [16, 24, 31, 43, 44].

Studies using PMA_{xx} evaluated infectious HAdV particles (serotypes 2, 5, 12, and 40) in samples of phosphate-buffered saline, food (mollusc and sausage), and environmental (soil, sewage, and freshwater from the urban river), thus demonstrating a reduction ranging from 0.42 to 4 log [19, 20, 22, 24, 27, 45]. PMA or PMA_{xx} were also efficient in detecting another viruses, enteric or not, including SARS-CoV-2 with intact capsids in clinical, food, and environmental matrices [16, 31, 32, 43, 46–54] providing valuable data for environmental surveillance.

Conclusion

The presence of potentially infectious viral particles depends on each sampling, regardless of the matrix, although a higher concentration of damaged particles was evident in sewage and seawater samples. Association of this genomic intercalant with qPCR can become a quick analytical tool for

screening for infectious HAdV in environmental samples, including sea and brackish water.

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Authors' contributions Conceptualization: Lorena da Graça Pedrosa de Macena, and Marize Pereira Miagostovich. Data curation: Lorena da Graça Pedrosa de Macena, Natália Maria Lanzarini and Adriana Gonçalves Maranhão. Formal analysis: Lorena da Graça Pedrosa de Macena, Natália Maria Lanzarini, and Marize Pereira Miagostovich. Investigation: Lorena da Graça Pedrosa de Macena, and Joseane Simone de Oliveira Pereira. Methodology: Lorena da Graça Pedrosa de Macena, Joseane Simone de Oliveira Pereira, Jansen Couto da Silva, Adriana Gonçalves Maranhão, and Fernando César Ferreira. Supervision: Marize Pereira Miagostovich. Visualization: Lorena da Graça Pedrosa de Macena and Marize Pereira Miagostovich. Writing—original draft: Lorena da Graça Pedrosa de Macena. Writing—review and editing: Lorena da Graça Pedrosa de Macena and Marize Pereira Miagostovich. Funding acquisition: Marize Pereira Miagostovich. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare that they have no conflict of interest.

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