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STOTEN-21758; No of Pages 6

Science of the Total Environment xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Optimization of the skimmed-milk flocculation method for recovery of adenovirus from sludge

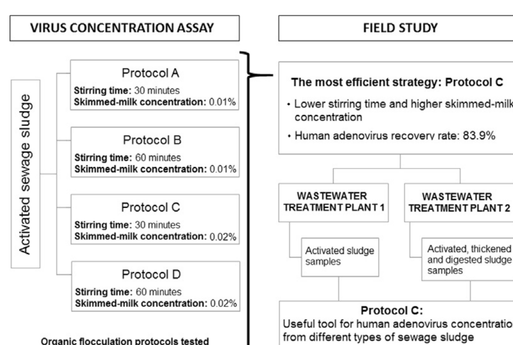
Andrêssa S.F. Assis [a,*], Marcelo Henrique Otenio [b], Betânia P. Drumond [a,1], Tulio M. Fumian [c], Marize P Miagostovich [c], Maria Luzia da Rosa e Silva [a]

[a] Laboratory of Virology, Microbiology Department, Institute of Biological Science, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil
[b] Brazilian Agricultural Research Corporation, Embrapa Dairy Cattle, Juiz de Fora, Minas Gerais, Brazil
[c] Laboratory of Comparative and Environmental Virology, Oswaldo Cruz Institute, Rio de Janeiro, Rio de Janeiro, Brazil

HIGHLIGHTS

- Increase in skimmed-milk concentration improved adenovirus recovery rate from sludge.
- Increase of the stirring time in glycine buffer decreased adenovirus recovery rate.
- Adenovirus detection in different sludge types proved the efficiency of the method.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 31 October 2016
Received in revised form 6 January 2017
Accepted 7 January 2017
Available online xxxx

Editor: Simon Pollard

Keywords:
Sewage sludge
Wastewater treatment plant
Organic flocculation
Human adenovirus
Internal control
Quantitative PCR

ABSTRACT

Return of treated sludge to the environment poses concerns and has stimulated the development of studies on viral monitoring in this matrix, in order to assess its potential risks for public health. Human adenovirus (HAdV) has been identified as a putative viral marker of faecal contamination due to its stability and resistance to the sewage treatment process. The aim of this study was to optimize the organic flocculation procedure in order to establish an appropriate methodology for HAdV recovery from sewage sludge samples. Four protocols (A-D) have been proposed, with changes in the initial sample dilution, in the stirring time and in the final concentration of skimmed-milk. A single sludge sample was obtained in Wastewater Treatment Plant (WWTP) and divided into aliquots. In each protocol, three aliquots were inoculated with HAdV and bacteriophage PP7 and a non-inoculated one was used as negative control. Viral load and recovery rate were determined by quantitative PCR. HAdV recovery rate varied between the protocols tested ($p = 0.016$) and the best result was obtained through the protocol C. In order to confirm this result a field study with activated, thickened and digested sludge samples was carried out. Different types of sludge were obtained in two WWTPs and processed using protocol C. HAdV was detected in all samples, with a similar or higher viral load than those obtained with other concentration techniques already applied to sludge. Protocol C proved to be really efficient, with the advantage of showing low cost and practicability in routine laboratories.

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* Corresponding author.
E-mail address: andressasilvino@yahoo.com.br (A.S.F. Assis).
[1] Present address: Betânia P. Drumond, Laboratory of Virus, Microbiology Department, Institute of Biological Science, Federal University of Minas Gerais, Minas Gerais, Brazil.

http://dx.doi.org/10.1016/j.scitotenv.2017.01.045
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Please cite this article as: Assis, A.S.F., et al., Optimization of the skimmed-milk flocculation method for recovery of adenovirus from sludge, Sci Total Environ (2017), http://dx.doi.org/10.1016/j.scitotenv.2017.01.045

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1. Introduction

Sewage sludge is a waste product generated by the sedimentation of suspended solids during the process of wastewater treatment. This by-product has a complex nature due to the large accumulation of organic compounds, macro and micronutrients, as well as traces of heavy metals (Singh and Agrawal, 2008; Rock et al., 2010). In general, the sludge treatment involves thickening, stabilizing the organic matter, dehydration and disinfection. The final disposal of treated sludge varies among incineration, disposal in landfills and agricultural applications (Singh and Agrawal, 2008).

The sludge also contains many pathogens, especially of faecal origin, such as bacteria, protozoa, helminths, and viruses (Sidhu and Toze, 2009). Most human enteric viruses present in the raw sewage is adsorbed to organic compounds and suspended solids, becoming therefore, part of the sludge (Sidhu and Toze, 2009; Wong et al., 2010). A large proportion of such viruses can be inactivated and/or eliminated during the treatment (Templeton et al., 2008). However, studies have demonstrated that enteric viruses can withstand the processes used in sludge stabilization (Viau and Peccia, 2009; Wong et al., 2010). Thus, the sludge disposal in landfill, as well as its agricultural reuse, can lead to the contamination of surface water, ground water and soil, creating a potential risk for human exposure (Sidhu and Toze, 2009). This exposure can occur either by direct contact by handling of the sludge and/or the soil where the waste was applied or by aerosols produced during the application or indirectly, by the consumption of food grown in soil fertilized with sludge or animals which grazed in these lands or intake of water contaminated by the infiltration of pathogens into the soil or by sludge dragging due to rain water (Sidhu and Toze, 2009).

Human enteric viruses are involved in outbreaks and sporadic cases of diarrheal diseases caused by the ingestion of contaminated water and food (Rodriguez-Manzano et al., 2014; Braeye et al., 2015). The explosive character and quick spread of the outbreaks may be associated with the increased amounts of viral particles secreted in the faeces (up to 10^{11} particles/gram) and the small infective dose required to trigger an infection (about 10 infectious units) (Fong and Lipp, 2005; Gall et al., 2015). In addition, these agents can withstand a wide range of pH (pH 3 to 10) in the environment and may remain infectious for up to 120 days in freshwater and wastewater, and 100 days in soil at temperatures ranging from 20 to 30 °C (Fong and Lipp, 2005).

Among the enteric viruses, human adenovirus (HAdV) are widely detected throughout the year and show stability in both domestic wastewater and environmental samples, in addition to withstand water and sewage treatment processes (Pusch et al., 2005; Bofill-Mas et al., 2006; Albinana-Gimenez et al., 2009; Okoh et al., 2010). Due to these characteristics, such viruses have been considered as being a likely viral marker of human faecal contamination in aquatic environments.

The aim of this study was to optimize the organic flocculation method using skimmed-milk for recovering HAdV from sludge samples. This method, first described for virus concentration in seawater, has been adapted for different matrixes (Calgua et al., 2008, 2013a, 2013b; Melgaço et al., 2016). The viral concentration technique based on organic flocculation shows advantages over other methods, such as membrane filtration and ultracentrifugation. The organic flocculation procedure is easy and fast to be carried out, it requires a simple laboratory infrastructure, thus eliminating costly equipment, in addition to the low cost of the inputs necessary to be performed.

2. Materials and methods

2.1. Virus samples

HAdV type 5 (ATCC® VR1516™) was propagated in Hep-2 cells (Ferreira et al., 2014) and the stocks of cell culture supernatant were used as inoculum (positive control).

Bacteriophage PP7 (ATCC 15692-B2), kindly provided by Dr. Verónica Rajal (Salta University, Argentina), was produced by culture in the host *Pseudomonas aeruginosa* (ATCC 15692) using a previously described protocol (Rajal et al., 2007). PP7 was used as an internal control in all the experiments in order to avoid false negative results associated with the enzyme inhibition in the molecular detection reactions.

2.2. Study area description

Sewage sludge samples were collected in two Wastewater Treatment Plants (WWTP), in Brazil. The WWTP1 is located in the municipality of Juiz de Fora, state of Minas Gerais. The plant operates through a secondary treatment known as activated sludge system with prolonged aeration process. In this system, the sludge is stabilized within an aeration tank, requiring only a dehydration treatment stage before sludge final disposal into sewage. The WWTP1 receives domestic sewage from approximately 60,000 inhabitants, besides landfill leachate, wich is rich in inorganic and organic pollutants. Activated sludge (AS) samples from WWTP1 were used for virus concentration assay optimization and for field studies.

The WWTP2 is located in the metropolitan area of Rio de Janeiro city, state of Rio de Janeiro. This plant receives urban sewage from around 1.5 million people living in the Central and North areas. The WWTP2 employs a secondary treatment using conventional activated sludge process. Activated Sludge (AS), Thickened Sludge (TS) and Digested Sludge (DS) samples were collected from this plant and used for field study.

All sludge samples were harvested in sterile polyethylene bottles and transported to the laboratory at 4 °C, where they were processed within 24 h.

2.3. Study design

In order to determine the most effective strategy to recover viruses from sewage sludge samples, four protocols based on organic flocculation, described by Calgua et al. (2013a), were tested (Fig. 1A). Then, the most effective protocol for virus concentration was used in a field study to investigate the presence of HAdV in sewage sludge samples from WWTPs (Fig. 1B).

2.3.1. Virus concentration assay

For virus concentration assay, a total of 240 mL activated sewage sludge was collected from the WWTP1, divided into four aliquots (60 mL) and processed by using different protocols: either A, B, C or D (Table 1). Each 60 mL aliquot was then subdivided into 15 mL aliquots, three of them were spiked with HAdV and PP7 (500 μL of each virus) and one uninoculated aliquot was used as negative control.

Briefly, the viruses in sludge sample (15 mL) were eluted by using 135 mL of glycine buffer 0.25 N, pH 9.5 (1:9, v/v). Samples were stirred for 30 min (protocols A and C) or 60 min (protocols B and D) (Table 1) in ice and centrifuged at 8000 ×*g* for 30 min at 4 °C. The supernatant (150 mL) was transferred to a new bottle and the pH was adjusted to 3.5 with HCl 6 N. The pre-flocculated skimmed-milk solution (1%, w/v) was prepared as previously described (Calgua et al., 2013a). This solution was added to the supernatant in order to obtain a final concentration of 0.01% (w/v) (protocols A and B) or 0.02% (w/v) (protocols C and D) of skimmed-milk (Table 1). Samples were stirred for 8 h at room temperature and flakes were pelleted by centrifugation at 8000 ×*g* for 30 min at 4 °C. The supernatants were carefully removed and the pellet was dissolved in 1000 μL of phosphate buffer (pH 7.5). The final viral concentrates were stored at -80 °C until processed, as follows (items 2.4 and 2.5).

2.3.2. Field study

The most effective protocol for virus recovery was applied to field samples. Eight activated sludge samples (AS1-AS8) were collected from the WWTP1 (two campaigns per week during February/2014). In parallel, two samples of activated sludge (AS9-AS10), thickened

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