Evaluation of virus recovery methods and efficiency of tannin-derived coagulants in removing total coliforms, E. coli and enteric viruses in effluents of a domestic sewage treatment plant

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**ABSTRACT**

In the present study, nine coagulants having potential to be used for sewage treatment were compared to assess their efficiency in removing total coliform bacteria, *Escherichia coli* and adenovirus. The coagulants tested were metallic and organic and their efficiency was compared when treating samples of raw and treated sewage (activated sludge). Before the efficiency tests of the coagulants, viral concentration methods were compared. Coagulation tests were carried out by using the jar-test system and the doses used ranged from 100 ppm to 1,000 ppm. Viral DNA was extracted and subjected to real-time polymerase chain reaction (qPCR) using primers for the gene of AdV hexon. Aluminum sulfate (1,000 ppm) presented the best results for raw sewage among metal coagulants whereas Acquapol® C118 and WW (1,000 ppm) had the most satisfactory results among organic coagulants, both reducing up to 7 logs for coliforms and 4 logs for virus. For the treated effluent, FeCl2 (1,000 ppm) presented best results for metal coagulants, whereas, from organic coagulants, the best removal rates were for Acquapol® 893/11 (1,000 ppm), both reducing up to 3 logs for coliforms and 4 logs for virus.

**Key words** | adenovirus, coagulants, tertiary treatment, viral concentration

**INTRODUCTION**

Human adenovirus (HAdV) is the most prevalent human virus in sewage (Rigotto *et al.* 2010) and is frequently found in environmental samples contaminated by human feces. The main means of contamination of the water bodies are leakage or discharge of sewage and urban or rural outflow. HAdVs are also often found in urban rivers or polluted coastal waters where they persist for up to 4 months. Formiga-Cruz *et al.* (2005) found that HAdV is excreted throughout the year in larger numbers than enteric viruses (EV) and hepatitis A virus (HAV); besides, among these viruses, HAdV was found to be more prevalent in water and oyster samples, and its viability percentage in water samples was also higher. The presence of HAdV has already been reported in surface waters contaminated by untreated sewage in the city of Porto Alegre (Vecchia *et al.* 2012).

Previous studies showed that AdV is more stable than many EV such as poliovirus or HAV in tap water or seawater, being also relatively resistant to chlorination and filtration used during conventional water treatment. AdV is considerably more resistant than other viruses to ultraviolet (UV) light elimination as well, which is sometimes used as tertiary treatment. Treatment with UV radiation is the option that is receiving the most attention. Although primary and secondary sewage treatment processes can eliminate 90–99.9% of enteric microorganisms, tertiary treatment, such as filtration, can still reduce up to 90–99% of these organisms in primary or secondary treated water. In spite of this, the treated sewage can still contain high microbial numbers (Leong 1985; Rajala *et al.* 2003; Jiang *et al.* 2006).

Viruses are found in the environment in small quantities and, as they are obligate intracellular parasites, they do not multiply in the environment, which requires the use of viral concentration methods for large amounts of sample. A wide
A variety of concentration methods are available and the choice of which one to use depends on the sample type, the virus to be detected and even on the financial resources of the laboratory. Some examples are ultrafiltration (Soule et al. 2000; Rajala et al. 2003), immunomagnetic separation (Myrmel & Wasteson 2000), ultracentrifugation (Formiga-Cruz et al. 2005), viral adsorption using amino-fractionalized silica particles (Chen et al. 2006), organic flocculation (Vantarakis & Papapetropoulou 1999), precipitation with multivalent salts (also called inorganic flocculation) (Farrah & Bitton 1999), precipitation with polyethylene glycol (PEG) (Lewis & Metcalf 1988) and use of simple microfiltration membranes or cartridges, being either negatively or positively polarized, with significant differences in the use of these. Due to the type of sample of this study, concentration was restricted to the use of the precipitation method through PEG 6000, as described by Lewis & Metcalf (1988), with minor modifications.

Domestic effluent treatment systems in Brazil are composed by the integration of treatment methods. The system is usually divided into primary (preliminary), secondary and possibly tertiary treatment. As a general rule, physical-chemical treatment (tertiary) is used to remove phosphorus, and chlorination is used to remove pathogens, focused on the removal of Escherichia coli. Modern use of coagulants for water treatment began more than a century ago, when ferric chloride and aluminum sulfate were used as coagulants in large-scale water treatment works. Some studies have shown the possibility of using coagulating agents with oxidizing power such as ferrate ion, which could favor the oxidation of chemical species simultaneously to the coagulation process. The main coagulant products are: aluminum sulfate, aluminum polychloride, iron sulfate, ferrous chloride and organic coagulants. Aluminum sulfate is the most widely used coagulant because of its low cost and large scale production. It is a synthetic polymer made from aluminum hydrate. Some examples of coagulants derived from plant species are from Moringa oleifera and Opuntia ficus-indica (Sánchez-Martín et al. 2010), tannins that are polyphenol compounds found in a large variety of higher plants and chemically considered as substances capable of precipitating proteins. The use of tannins as a coagulant/flocculant is increasing more and more because, compared to metallic salts, it is a product of renewable origin, which generates less sludge, besides being a non-hazardous sludge, and with greater ease of elimination, which contributes to a cleaner effluent treatment process (Piantá 2008). Acquapol®, a tannin-based product tested in this study, which is modified by a physicochemical process with a high flocculating power, is obtained from the bark of Acacia mearnsii (Acacia negra), a very common tree in Brazil with a high concentration of tannins. Due to this property, it becomes a potential coagulant and flocculant, since it has an anionic behavior in solution.

The coagulation process destabilizes colloidal impurities, transforming small particles into large aggregates, and absorbs dissolved organic materials on the aggregates, which can then be removed by sedimentation and filtration.

This work evaluated the efficiency of removing microorganisms (coliforms and adenovirus) from domestic sewage by physical-chemical process of coagulation/flocculation and sedimentation with the use of metallic coagulants and organic coagulants of vegetal origin, such as Acquapol®. The study aimed to compare the performance of coagulants in the treatment of raw sewage and sewage previously treated by activated sludge process, both collected at a sewage treatment plant (STP) in the metropolitan region of Porto Alegre, in the southernmost state of Brazil. In addition, studies were carried out to evaluate the best methodology for viral extraction in the samples.

**METHODS**

**Sample collection**

The samples of domestic sewage were provided by Companhia Riogradianse de Saneamento (CORSAN) and were collected in an STP of a municipality of the metropolitan region of Porto Alegre, in the state of Rio Grande do Sul (Brazil), with a flow of 22,464 m³/day. A total of 50 L of sewage was collected after preliminary treatment (raw sewage) and after secondary treatment by activated sludge (treated sewage). Seven collections were carried out, of which four were collected in the month of May and one in the month of August 2011 and two in the months of March and May 2012. Regarding the standardization of detection assays and jar-tests, all were made from an initial volume of 500 mL.

**Jar-test**

Samples were submitted to jar-test using different coagulants on the same day of collection: 2 L samples of sewage were mixed to the coagulant/flocculant for 1 min at 120 rpm (rapid mixing) and 5 min at 50 rpm (slow mixing), followed by sedimentation/decantation (40 min).
Positive controls

All experiments were made using a prototype viral strain of human adenovirus subtype 2 (HAdV-2). Viruses were cultivated in A549 cells following standard procedures.

Evaluation of the viral detection methodology

In order to compare viral detection methodologies, the following coagulants and concentrations were tested in the first sampling for raw sewage (May 2011): Ferrous Aluminum Sulfate 300 ppm, Ferrous Aluminum Sulfate 350 ppm, Acquapol® WW 300 ppm, Acquapol® WW 350 ppm, Acquapol® OF 18, Acquapol® C1 18, Acquapol® 893/11 and Acquapol® T832, all derived from tannin. All coagulants were supplied by the Aquaquímica S/A company (Estância Velha, Brazil). The dosages used were between 100 ppm and 1,000 ppm, which were chosen based on previous tests.

For the evaluation of HAdV, the technique of extraction without previous concentration was used, which presented the best results when evaluating viral extraction methodologies.

HAdV-2 were added to the sample previously to coagulation and then quantified to take into account the matrix effect. Afterwards, the aforementioned coagulants were used, being carried out in a new extraction and quantification of the samples after the treatment by the coagulant. The quantifications (infectious doses) of HAdV-2 detected before and after the use of these coagulants were used for the calculation of virus removal. Three repetitions were used for each test.

Nucleic acid extractions

Nucleic acid extraction was performed using the RTP DNA/RNA Virus Mini Kit (Invitek®) for all samples tested, using 400 μL of each sample after the concentration steps or without previous concentration.

Polymerase chain reaction

Samples used to evaluate viral recovery methods were performed by PCR. They were submitted to amplification procedures with primers described in the literature, which encode the conserved region of the HAdV hexon protein gene, called VTB2-HAdVC forward primer (5′- GAGACG TACTTCAGCCTGAAT-3′) and VTB2-HAdVC reverse primer (5′- GATGAACCGCAGCTCAAA-3′) (Wolf et al. 2010). The GoTaq Green Master Mix kit (Promega, USA) (25 μL) was used, along with 18 μL of nuclease-free water, 1 μL of each primer (20 μM), and 5 μL of the extracted, resulting in a final volume of 50 μL of solution. PCR was...
performed using 40 cycles, each cycle consisting of 1 min at 94 °C for denaturation, 1 min at 55 °C for annealing and 1 min at 72 °C for extension. The product was stained with non-toxic SYBR Safe® stain, submitted to 2% agarose gel electrophoresis and visualized under UV light.

**Real-time PCR (qPCR)**

After the choice of viral recovery method, all samples were performed only by real-time PCR (qPCR), reactions were performed on a Bio-Rad iQ5 thermocycler (Bio-Rad, USA) using the MyiQ™ Multi-Color Real-Time PCR Detection System with iQ™ 5 optical system software version 2.1. The Platinum® SYBR® Green qPCR Supermix-UDG commercial kit (Invitrogen, USA) was used, following methodology recommended by the manufacturer. The primers used have been described in the literature, which encode the conserved region of the HAdV hexon protein gene, termed VTB2-HAdVC forward primer (5'-GAGACGTACTTCAGCCCTGAAT-3') and VTB2-HAdVC reverse primer (5'-GATGAACCGCACTCAGCCTCAAA-3') (Wolf et al. 2010). For qPCR, 40 cycles were used, each cycle consisting of 1 minute at 94 °C for denaturation, 1 minute at 55 °C for annealing and 1 minute at 72 °C for extension. All samples (nucleic acids) were previously diluted (1:10) in order to minimize the action of inhibitors. The typical limit of detection was found to be 40 to 60 genome copies per reaction (Staggemeier et al. 2018). The genomic quantification of the present work represents the number of genome copies found in a volume of 500 mL of sample (as used for the whole experiments).

**Detection of coliforms**

The Colilert system (IDEXX® Labs) was used for simultaneous detections, specific and confirmatory identifications of total coliforms and *E. coli* following manufacturer’s instructions. Colilert analyzes were performed before and after exposure to coagulants to calculate total coliforms and *E. coli* removal.

**RESULTS**

**Evaluation of viral extraction methodologies**

In the comparison of viral extraction methodologies, 50% of the samples with concentration using PEG without dilution presented inoculated HAdV, whereas in the diluted samples the HAdV was detected in only 10% of the cases. In the samples in which extraction without previous concentration was performed, inoculated HAdV was detected in 100% of the samples, as shown in Table 1.

**Efficiency of coagulants**

The mean removal rates of total coliforms, *E. coli* and adenovirus were determined for the raw sewage samples from all collections after jar-test assays with different coagulants. The results are described in Table 2.

We observed that, for the raw effluent, the metal coagulant that stood out the most was aluminum sulfate with a dosage of 1,000 ppm, with a mean removal rate of the collections for total coliforms, *E. coli* and adenovirus of 100%, 99.37% and 99.72%, respectively. Among the tannin-derived coagulants, the most efficient were Acquapol® C1 18 (mean removal rate for total coliforms of 99.66%, for *E. coli* 99.61% and adenovirus 96.22%) and Acquapol® WW (mean removal rate for total coliform collections was 99.01%, for *E. coli* 99.19% and for adenovirus 88.85%), both at a dosage of 1,000 ppm. All of them eliminated up to 7 logs of coliforms and 4 logs of HAdV.

The results of jar-tests performed with effluent samples previously submitted to treatment by activated sludge are described in Table 3.

The metal coagulant with the best mean removal rate for the sewage with activated sludge treatment was the ferric chloride at a dosage of 1,000 ppm (total coliforms 100%, *E. coli* 100% and adenovirus 99.06%), whereas among the organic coagulants, Acquapol® 893/11 had a better mean removal rate (total coliforms 96.69%, for *E. coli* 99.19% and and adenovirus 88.58%). Both coagulants eliminated up to 3 logs of coliforms and 4 logs of HAdV.

**DISCUSSION**

**Viral extraction**

The efficacy of a concentration method depends on many variables, such as the amount of virus and the nature and...
### Table 2 | Average removal rates of total coliforms, *E. coli* and adenovirus for raw sewage samples

<table>
<thead>
<tr>
<th>Coagulant</th>
<th>Total coliforms (%)</th>
<th><em>E. coli</em> (%)</th>
<th>Adenovirus (%)</th>
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<tr>
<td>Al Sulfate 1,000 ppm</td>
<td>100</td>
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<td>Acquapol® C1 18 1,000 ppm</td>
<td>99.66</td>
<td>99.61</td>
<td>96.22</td>
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<tr>
<td>Acquapol® WW 1,000 ppm</td>
<td>99.01</td>
<td>99.19</td>
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</tr>
<tr>
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<td>98.91</td>
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<td>Ferrous Al Sulfate 1,000 ppm</td>
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<td>81.75</td>
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<td>Acquapol® T832 1,000 ppm</td>
<td>97.13</td>
<td>96.50</td>
<td>81.74</td>
</tr>
<tr>
<td>PAC-12 1,000 ppm</td>
<td>96.82</td>
<td>94.85</td>
<td>79.07</td>
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<tr>
<td>Acquapol® 893/11 1,000 ppm</td>
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<td>94.83</td>
<td>58.42</td>
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<td>Al Sulfate 100 ppm</td>
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<td>17.90</td>
<td>49.80</td>
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<tr>
<td>Acquapol® T832 100 ppm</td>
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<td>0</td>
<td>49.73</td>
</tr>
<tr>
<td>PAC-12 100 ppm</td>
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<td>0</td>
<td>5.83</td>
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### Table 3 | Average removal rates of total coliforms, *E. coli* and adenovirus for treated sewage samples

<table>
<thead>
<tr>
<th>Coagulant</th>
<th>Total coliforms (%)</th>
<th><em>E. coli</em> (%)</th>
<th>Adenovirus (%)</th>
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</thead>
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<td>FeCl₂ 1,000 ppm</td>
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<td>PAC-12 1,000 ppm</td>
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<td>90.36</td>
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<tr>
<td>Al Sulfate 1,000 ppm</td>
<td>9</td>
<td>88.33</td>
<td>30.96</td>
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<td>5</td>
<td>39.47</td>
<td>27.08</td>
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<tr>
<td>Acquapol® 893/11 100 ppm</td>
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<td>0</td>
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</table>
the amount of the environmental sample (Rigotto et al. 2010). In the studies of Karamoko et al. (2005), the adsorption-elution concentration method (Myrmel et al. 1999) was used for the detection of HAdV in samples of urban sewage, in which, of the 15 samples analyzed, only three samples were positive for HAdV.

In the recent work by Calgua et al. (2013) the most effective method for sewage concentration was a skimmed milk organic flocculation process, among ultracentrifugation, ultrafiltration and lyophilization methods. In the present work, it was verified that, for the type of sample analyzed, the most effective method was not using a concentration step, performing direct extraction of the sample. No description of direct extraction without concentration was found in the literature for water samples, which gives importance to this study in demonstrating that the use of several steps for the concentration of a water sample can often cause false negatives.

**Efficiency of coagulants**

Pathogenic microorganisms such as viruses are often found in treated effluents because such treatment is not the most correct or appropriate, and the presence of such pathogens may pose a great risk to public health. Effective monitoring of these pathogens is, therefore, important, especially in urban settings. Most cases of enteric virus infections originate from contaminated sources of drinking water, recreational waters, and food contaminated by sewage waters and sewage effluents (Svraka et al. 2007). The processes used to treat wastewater, such as activated sludge, oxidation ponds, activated charcoal treatment, filtration, coagulation and chlorination, have the capacity to eliminate between 50% and 90% of the viruses (Cloette et al. 1998), allowing a significant viral load to be released into the environment through effluents. The performance of sewage/water treatment systems is measured using bacterial indicator organisms. However, the presence of viral agents in water resources is observed even in the absence of bacteria, making this, which is the only monitoring tool used for the presence of microorganisms, insufficient (Okoh et al. 2010).

In the present study, the best results in the treatment of raw and treated sewage were achieved by metallic coagulants (aluminum sulfate and ferric chloride, respectively). However, tannin-based coagulants showed similar results: both metallic and organic removed 7 logs of coliforms and 4 logs of HAdV. Studies have shown that the secondary treatment with activated sludge can remove up to 5.4 logs of total coliforms and 5.7 of E. coli (Zhang & Farahbakhsh 2007), requiring alternatives to improve effluent quality. UV irradiation is listed by the United Stated Environmental Protection Agency (US-EPA) as a tertiary treatment for viral clearance (US-EPA 1990), as it is able to effectively eliminate viruses, bacteria and parasites without generating by-products or chemical residues. However, the efficiency of UV disinfection depends on the conditions of the water and it is possible for the affected microorganisms to be repaired by enzymatic photoreactivation (Salgot et al. 2002). In the study conducted by Gerba et al. (2002) it was possible to inactivate 99.9% of HAdV type 2 using a dose of 119 mW/cm². Studies by Thurston-Enriquez et al. (2003) showed the use of a tertiary method by exposing the sample to ozone doses, obtaining results for removal of HAdV of up to 3.55 logs using doses of 0.30 mg/L.

Although aluminum sulfate showed the best results for raw sewage, reducing up to 7 logs of coliforms and almost 3 logs (99.72%) of HAdV, the use of inorganic salts in the treatment of sewage, such as aluminum chloride or sulfate itself is controversial due to the possible impact of aluminum on human health. Aluminum, depending on the dosage is toxic, causing neurodegenerative diseases such as dementia and Alzheimer’s disease (Thakur & Choubey 2014).

Coagulants must be biodegradable to be safe for human health, such as those derived from plant species, which produce smaller amounts of sludge than aluminum sulfate and are easily biodegradable (Yin 2010). In addition, these coagulants adsorb dissolved metals in water and eliminate or reduce toxicity from sources such as cyanophytes or chlorophyll bacteria. The sludge resulting from this treatment can also be used as raw material for the production of organic fertilizers with slow and controlled release of nitrogen.

There are recent works proposing the substitution of synthetic inorganic or organic polymers by organic cationic polymers prepared from products of plant origin, such as tannins, in the sustainable support to water treatment (Mangrich et al. 2014). According to Mangrich et al. (2014), tannin-derived coagulants are suitable to meet the recommendations of bodies such as the UN and programs such as Gii in water and sewage treatment. However, Özacar & Sengil (2002) reported better results in water treatments using tannin as a flocculation aid with aluminum sulfate than tannin as the primary coagulant.

Given the need to implement new technologies for the treatment of water and effluents adapted to the constraints of developing countries, the potential of plant-based
Coagulants as sustainable is increasingly recognized. Nevertheless, the use of these coagulants for the treatment of effluents has been limited to academic investigations, which had indicated their good potential for effluent treatment (Vijayaraghavan et al. 2011). Tannins, in addition to coagulant properties, have disinfectant effects due to the formation of quinones, which can be used as algicide, fungicide and antibacterial, thus reducing or eliminating the content of microorganisms capable of causing diseases in humans.

As observed in this study, the tannin-based coagulants also have viral elimination capacity, with the potential to make water treatment processes and effluents that seek such results less costly. In the United States, a treatment process is considered ideal when there is a reduction/ inactivation of 4 logs (99.99%) of virus in the treated effluent (Florida Department of Environmental Protection 2009), and this was the exact amount eliminated by the metallic coagulants and tannin-based coagulants in at least one of the seven collections performed, demonstrating their potential ability to remove viral agents as recommended by US agencies. There is a shortage of comprehensive studies comparing the efficiency of plant-based coagulants and metallic coagulants or synthetic polymers, possibly being one of the factors that inhibit their application in effluents (Vijayaraghavan et al. 2011). Thus, the substitution of metallic or synthetic coagulants for alternative coagulants of plant origin should be progressive (Thakur & Choubey 2014).

CONCLUSION

In the comparison of viral extraction methodologies, the best results were obtained in samples where extraction was performed without prior concentration, detecting HAdV in all samples.

In the comparison of coagulants for the removal of coliforms and HAdV, no coagulant obtained the highest removal rate for all the microorganisms in question in both types of samples, raw and treated. In addition, for the raw effluent, the metal coagulant that presented the best results was aluminum sulfate with a dosage of 1,000 ppm, whereas Acquapol® C1 18 and Acquapol® WW, both using a dosage of 1,000 ppm, were highlighted among the organic ones. For the treated effluent samples, the metal coagulant with the best removal rate was FeCl3, with a dosage of 1,000 ppm, whereas among the organic coagulants Acquapol® 893/11 showed a higher mean removal rate. Elimination of up to 4 logs (99.99%) of virus in this study demonstrates that both metal and organic coagulants are able to reduce a considerable amount of these pathogens.

Research on the use of coagulants derived from plants is of great interest since they are products with lower environmental impact than metallic coagulants. However, there is still a need for further research for its full use, and is essential to verify the possible mutagenic substances in these coagulants, especially when used for the treatment of water or sewage.

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