UNIVERSIDADE FEEVALE
PROGRAMA DE PÓS-GRADUAÇÃO EM QUALIDADE AMBIENTAL
MESTRADO EM QUALIDADE AMBIENTAL

MERIANE DEMOLINER

RASTREAMENTO DE FONTES DE CONTAMINAÇÃO MICROBIANA EM PEQUENAS FAZENDAS POR DIFERENTES MÉTODOS EMPREGADOS PARA DETECÇÃO DE ADENOVÍRUS

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Dissertação apresentada ao Programa de Pós-Graduação em Qualidade Ambiental como requisito para a obtenção de título de mestre em Qualidade Ambiental

Orientador: Prof. Dr. Fernando Rosado Spilki

Co-orientadora: Prof. Drª. Andréia Henzel

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Dissertação de mestrado aprovada pela banca examinadora em 21 de Fevereiro de 2019, conferindo ao autor o título de mestre em Qualidade Ambiental.

Componentes da Banca Examinadora:

Prof. Dr. Fernando Rosado Spilki
(Orientador)
Universidade Feevale

Profª. Drª. Juliane Deise Fleck
Universidade Feevale

Profª. Drª. Ana Cláudia Franco
Universidade Federal do Rio Grande do Sul

RESUMO

Os virus entéricos, em especial os adenovírus (AdV), são abundantes no meio ambiente e frequentemente encontrados em matrizes aquáticas ou no solo. O presente estudo almeja expandir o conhecimento sobre a contaminação fecal de humanos e animais usando os AdV como bioindicadorES em diferentes fontes de águas no contexto zona rural de municípios do Vale dos Sinos, avaliando também a infecciosidade e comparando as técnicas usadas para detecção de Mastadenovírus humano (*Human mastadenovirus*, HAdV). Para isso foram coletadas 124 amostras de água, sendo 86 subterrâneas e 38 superficiais. As coletas ocorreram nos meses de novembro e dezembro de 2015, em 34 propriedades rurais de 11 municípios localizados ao longo da Bacia Hidrográfica do Rio dos Sinos – RS. Além da contagem de *Escherichia coli* através do kit Colilert, as amostras foram submetidas a ensaios de detecção e caracterização de AdV por diferentes métodos (qPCR, multiplex-qPCR, and Nested-PCR) e, testes de infecciosidade viral (ICC-qPCR). A *E. coli* foi detectada em 62,8% das amostras de águas subterrâneas com uma média geométrica de 16,7 MPN/100mL e, nas águas superficiais sua taxa de detecção foi de 68,4%, com média geométrica de $5,08x10^2$ MPN/100mL. Dos indicadores virais em águas subterrâneas o HAdV teve uma maior detecção, sendo ela 48,84%, seguido do Mastadenovírus canino (*Canine mastadenovirus,* CAV) 19,77%, Mastadenovírus bovino (*Bovine mastadenovirus,* BAdV) 17,44%, *Aviadenovírus* (AvAdV) 15,12%, e Mastadenovírus porcino (*Porcine mastadenovirus,* PAdV) 3,49%, e em águas superficiais foi encontrado os seguintes resultados: HAdV 44,74%, CAV 42,11%, BAdV 28,95%, por fim com menor taxa de detecção o PAdV e AvAdV, ambos com 13,16%. A quantificação em copias genômicas dos AdVs variou de $9,40\ x10^4$ a $5,54x10^{10}$cg/L. Também foi detectado HAdV-C infeccioso em onze amostras (12,7%) das águas subterrâneas e sete (18,4%), nas superficiais. Notou-se um aumento na sensibilidade e, consequentemente no número de amostras positivas, ao utilizar mais de um ensaio para detecção de HAdV. O estudo mostra que o ambiente estudado sofre através da ação antrópica, onde a falta de saneamento básico é um importante percursor da contaminação fecal encontrada nas águas, enfatizando a importância de medidas de remediação e prevenção da contaminação do solo e da água, visando uma redução na degradação ambiental e no risco que a água representa à saúde humana e animal.

Palavras-chave: Adenovírus. Bioindicador. Contaminação fecal. Águas subterrâneas. Águas superficiais. Área rural.

ABSTRACT

The enteric viruses, especially the adenovirus (AdV) are abundant in the environment and frequently found in soil and water. The aims are expanded knowledge about the fecal contamination by humans and animals, using the AdVs as bioindicators in different water sources from rural areas of cities from Vale dos Sinos, also evaluating the infectivity and comparing the different techniques used to detect *Human mastadenovirus* (HAdV). To that, it was collected 124 samples, being 86 from groundwater and 38 of surface water. The collections were carried out in November and December of 2015, in 34 rural properties of 11 municipalities located along the Rio dos Sinos basin. The Escherichia coli count was realized by Colilert kit and, the samples were submitted the detection and characterization tests of AdV by different methods (qPCR, multiplex-qPCR and, nested-PCR). In addition, the viral infectivity was realized too by ICC-qPCR. The *E. coli* was detected in 62.8% of groundwater samples, with the geometric mean of 16.7 MPN /100mL and, in surface waters the detection rate was 68.4%, with the geometric mean of $5.08x10^2$ MPN/100. Amongst the viral indicator in the groundwater the HAdV was the with higher detection rate 48.84%, followed by *Canine mastadenovirus* (CAV) 19.77%, *Bovine mastadenovirus* BAdV 17.44%, *Aviadenovirus* AvAdV 15.12%, and *Porcine mastadenovirus* (PAdV) 3.49%. In surface water was found the following results: HAdV 44.74%, CAV 42.11%, BAdV 28.95% and finally, with less detection rate PAdV and AvAdV, both were found in 13.16%. The quantification of genomic copies per liter ranging from $9.40 x10^4$ to $5.54x10^{10}$cg/L. In groundwater samples, it was possible to observe 11.6% infectious samples, and in surface water, the total of infectious samples was 18.4%. The results showed a increased in the sensitivity and, consequently the number of positive samples when were used a set of techniques for HAdV detection.The study shows that the environment studied suffer on anthropic action, which the lack of basic sanitation facilities is an important forerunner for the fecal pollution found in the waters evaluated in that research. Thus, highlighting the importance of remediation and prevention measures to water and soil contamination, aiming the reduction in environmental degradation and in the health risk that the water represents for humans and animals.

Key words: Adenovirus. Bioindicator. Fecal contamination. Groundwater. Surface water. Rural area.

Sumário

1. INTRODUÇÃO GERAL

1.1 Contaminação das águas subterrâneas e superficiais

A contaminação de aquíferos é comum e pode ocorrer por diversos fatores (Howard et al., 2003; Gibson, 2014). O solo contaminado permite a lixiviação de contaminantes, especialmente patógenos de origem entérica, os quais são transportados por distancias significativas, migrando para as camadas mais profundas do solo, atingindo as águas subterrâneas através do processo de adsorção e dessorção (Knappett et al., 2012; Gotkowska-Płachta et al., 2015; Staggemeier et al., 2015).

Embora seja difícil elucidar a maneira que os vírus atingem as águas subterrâneas, atualmente existem confirmados quatro modelos conceituais do transporte do vírus até um aquífero (Bradbury et al., 2013).

I. Transporte por fluxo rápido ao longo do anel do poço e/ou através de adversidades relacionados com manutenção inadequada, como danificações na estrutura física, má instalação ou problemas de brecha no revestimento do poço.
II. Transporte através do aquitardo por fluxo de meios porosos.
III. Transporte por fluxo através de meios porosos ao redor da borda do aquitardo ou através de janelas ou brechas próximas ao aquitardo.
IV. Transporte por fluxo rápido através de fissuras no aquitardo ou através da conexão cruzada com poços próximos.

As águas superficiais também sofrem deterioração da sua qualidade através de fontes difusas, as quais são difíceis de identificar para a gestão adequada e planejamento de remediação. Entre elas, o escoamento urbano, florestal e agropecuária, sendo que o último inclui a contaminação por aplicação de dejetos no solo e o livre acesso do gado e de outros animais a rios e córregos (Do Amaral, 2004; Knappett et al., 2012; Staggemeier et al., 2015).

1.2 Parâmetros de qualidade e potabilidade da água

Atualmente, as bactérias pertencentes ao grupo dos coliformes termotolerantes são as principais utilizadas como indicadores de contaminação fecal em grande parte do mundo. No Brasil, a classificação e enquadramento dos corpos de água, são determinados pelo Conselho Nacional do Meio Ambiente (CONAMA), para águas superficiais: Resolução CONAMA de nº 357, 2005; e para subterrâneas: resolução CONAMA de nº 396, 2008. Ambas exigem apenas os coliformes termotolerantes como critério microbiológico para estabelecer essas definições (CONAMA, 2005; CONAMA, 2008). Assim também ocorre para as águas de abastecimento público, regulamentadas pela Portaria de número 2.014 de 2011 do Ministério da Saúde, que versa sobre a qualidade da água de consumo e seu padrão de potabilidade, adota apenas coliformes totais como indicador de eficiência de tratamento e Escherichia coli como indicador de contaminação fecal. O monitoramento de vírus entéricos nos pontos de captação é apenas recomentado, ou seja, não é obrigatório, e a análise para pesquisa de vírus e protozoários deve ser realizada somente quando há surtos de doenças diarreicas agudas ou outros agravos de transmissão fecal-oral. (Ministério da Saúde, 2011).

Muitos países, inclusive os Estados Unidos, empregam apenas coliformes termotolerantes como parâmetro microbiológico da água. Entre eles, *Enterococcus* e *E. coli* (EPA, 2014). Contudo, isso não é satisfatório, pois diferentes espécies de vírus entéricos podem ser encontradas em ambientes aquáticos que estão em conformidade com os padrões bacterianos, já que não há relação observável entre parâmetros bacteriológicos e a presença de vírus em água (Fong & Lipp, 2005; Hundesa et al., 2006; Dalla Vecchia et al., 2015). Além disso, a *E. coli* é um indicador inespecífico, ou seja, quando encontrada no ambiente não se consegue rastrear ou inferir sobre o seu hospedeiro, pois ela é encontrada invariavelmente tanto nas fezes de humanos como também em animais endotérmicos, diferentes dos indicadores de origem viral que são espécie-especificas (Gotkowska-Płachta et al., 2015).

1.3 Adenovírus

Os adenovírus (AdV), possuem formato icosaédrico e medem em torno de 60 a 100 nm. São vírus sem envelope e seu genoma é composto por DNA de fita dupla linear não segmentado (Santos & Soares, 2015). Suas características estruturais são as que lhes conferem maior estabilidade em condições adversas, incluido variações de temperatura, pH, tratamentos físicos e químicos. Adicionalmente, são capazes de utilizar enzimas da célula hospedeira para reparar danos causados ao DNA viral (Fong e Lipp, 2005). Além disso, a complexidade do capsídeo viral (constituído por várias proteínas e projeções), proporciona a ele uma maior proteção ao material genético, quando comparado com arquiteturas de capsídeo mais simples (Thurston-Enriquez et al., 2003). Também observa-se que tais características contribuem para sua reistência aos processos de filtração e cloração, que são os métodos mais empregados no tratamento de águas (Barardi et al., 2012).

O Mastadenovírus humano (*Human mastadenovirus*, HAdV), pertence ao gênero *Mastadenovirus* e, são divididos em sete espécies humanas (A – G) (ICTV, 2016). São vírus capazes de infectar uma variedade de tecidos e sistemas possibilitando diferentes tipos de infecções. Por sua vez, o intestino humano e o trato respiratório são os principais sítios de replicação (Santos & Soares, 2015). Um agente etiológico relevante de doenças diarreicas, sendo o segundo patógeno viral mais importante de gastroenterite infantil, depois do rotavírus (Fong et al., 2010). Os HAdV da espécie F, são os mais frequentemente relatados como causadores de infecções no trato gastrointestinal. No entanto, há outras espécies associadas com essas condições, como, HAdV-A, B, C, D e G (Santos & Soares, 2015; Rames et al., 2016). Além disso os HAdV da espécie C, responsáveis por doenças respiratórias, são capazes de se replicar no trato respiratório e podem ser eliminados através do intestino sem causar nenhum sinal de diarreia (Santos & Soares, 2015). A infecção por tal espécie é uma das causas mais comum de doenças respiratórias do trato superior de crianças, ocorrendo principalmente em crianças menores que 5 anos de idade (Roy et al., 2009; Cheng et al., 2015). Em geral, as espécies HAdV B, C e E causam infecções respiratórias, distribuídos globalmente e ocorrem ao longo do ano, atravessando todas as faixas etárias (Wang et al., 2013). Além das infecções já

citadas, os HAdV são responsáveis também por outros tipos, como do trato urinário (HAdV-A, B e C) e da conjuntiva (HAdV-B e E) (Santos & Soares, 2015).

Além dos seres humanos, os AdV também podem infectar uma ampla gama de espécies animais, incluindo mamíferos, pássaros, répteis, anfíbios e peixes (Benkő et al., 2002). O *Canine mastadenovirus* (CAV) pertence ao gênero *Mastadenovirus* e a espécie A, que possui dois genótipos, sendo eles CAV-1 e CAV-2 (ICTV¸ 2016). CAV-1 induz uma doença sistêmica, que tem como principal complicação o quadro de hepatite infecciosa canina, hoje uma manifestação clínica rara, mas usualmente grave. Já o CAV-2 replica principalmente no epitélio respiratório, levando muitas vezes ao quadro clínico de traqueobronquite característico do complexo da Tosse dos canis, em associação com Bordetella sp., e o vírus Parainfluenza canino 2. Além de cães domésticos, as infecções por CAV podem ocorrer em outras espécies como, coiotes, raposas vermelhas e lobos, podendo ser fatais nessas espécies silvestres (De Almeida Curi et al., 2010; Dowgier et al., 2016). O *Porcine mastadenovirus* (PAdV) faz parte deste mesmo gênero, mas são divididos em três espécies PAdV-A, PAdV-B e PAdV-C (ICTV, 2016), os quais geralmente não produzem patologias clinicamente graves, mas há relatos de casos de pneumoenterite e encefalite causadas por este vírus (De Motes et al, 2004). Já os Adenovírus bovino (BAdV), pertencem a dois gêneros distintos, sendo as espécies BAdV-A, BAdV-B e BAV-C correspondente o gênero *Mastadenovirus* e as espécies BAdV-D ao gênero *Atadenovirus* (ICTV, 2016). Alguns genótipos estão associados a quadros de queratoconjuntivite, doença febril aguda ou pneumoenterite, podendo outros ainda serem fatais (De Motes et al., 2004). O *Aviadenovírus* (AvAdV) pertencem ao gênero *Aviadenovirus* e são divididos em oito especies, essas responsáveis por infectar apenas aves. Eles vêm sendo associados a doenças como hepatite, bronquite, congestão pulmonar e edema em diferentes espécies de aves (ICTV, 2016).

1.4 Testes usados na detecção de vírus no ambiente

Atualmente a maioria dos testes para a detecção de vírus entéricos no ambiente são através da detecção de genoma por métodos moleculares, já que uma grande porção dos vírus entéricos não é detectada por métodos convencionais de

cultura celular, os quais são baseados na quantificação de partículas virais em células, dependendo de linhagem celulares especificas. (Donia et al., 2010; Girones et al., 2010). Por outro lado, os testes moleculares são ferramentas muito úteis na identificação de fontes de contaminação fecal. Já que para alguns vírus é o único método de detecção e quantificação. Nos últimos anos os protocolos disponíveis para aplicação dessas técnicas aumentaram e embora ainda significativos, os custos baixaram (Girones et al., 2010).

Dentre essas técnicas moleculares, encontra-se: a reação em cadeia da polimerase (PCR), que além da detecção de vírus entéricos, permite posteriores análises sequenciais que fornecem informações adicionais sobre as características filogenéticas das cepas identificadas (Girones et al., 2010). A PCR *nested*, além das possibilidades já mencionadas, apresenta uma maior sensibilidade, o que o torna bastante adequada para aplicação em amostras de água, já que muitas vezes o vírus está diluído nessas matrizes. (Girones et al., 2010; Kittigul et al., 2015). A reação em cadeia da polimerase quantitativa em tempo real (qPCR), ferramenta diferente da PCR convencional, permite estimar a concentração de patógenos na água. Considerando amostras ambientais, é uma técnica bastante sensível, o que diminui o risco de resultados falsos-negativo, uma vez que ela normalmente possui uma taxa de detecção maior que o PCR convencional (Staggemeier et al., 2015). Sendo uma técnica que quantifica, ainda, essa ferramenta apresenta outra vantagem, onde a partir da quantificação em combinação com levantamentos epidemiológicos é possível realizar estudos de avaliação de risco (Girones et al., 2010). Além das técnicas já mensionada, exite a PCR multiplex, que é muito utilizada para identificar patógenos virais em virologia diagnostica, e é uma excelente ferramenta para avaliar amostras ambientais, pois, é capaz de detectar e identificar de forma simultânea diversos agentes virais (Elnifro et al., 2000; Formiga-Cruz et al., 2015), proporcionando uma economia significativa em tempo e custo, quando comprado a protocolos separados de PCR monoplex, sendo assim, muito útil em estudos com amostragens numericas grandes (YAN, et al., 2003).

Mas recentemente, vem sendo cada vez mais utilizada a combinação de técnicas moleculares com cultivo celular, a fim de determinr a presença de particula infecciosas em amostras. Esse método tem se mostrado eficiente na detecção de adenovírus, enterovírus, astrovírus e reovírus em amostras ambientais (Rodríguez,

Pepper, & Gerba, 2009). Entretanto, ainda existem dificuldades de se obter modelo de cultura celular para a detecção de vírus que são transmitidos pela água (Rodríguez, Pepper, & Gerba, 2009; Donia, et al., 2010).

1.5 Descrição da região do estudo e da Bacia Hidrográfica do Rio dos Sinos

A Bacia Hidrográfica do Rio dos Sinos (BHRS), está localizada ao nordeste do Estado do Rio Grande do Sul, Brasil. Ela abrange 32 municípios e abastece cerca de 1,5 milhão de pessoas. Possui uma extensão de 196 km e uma área total de 3.820 km2. Suas nascentes estão localizadas no município de Caraá, na Serra Geral e sua foz em Canoas, desembocando no delta do rio Jacuí. A bacia atualmente é dividida em 3 sub-trechos distintos, sendo: (Dalla Vecchia, et al. 2015; FEPAM, 2014)

I. Trecho superior: representado por áreas de pequenas propriedades rurais, com baixas densidade populacional, onde predomina a agricultura e pequenas criações de gado leiteiro, suínos e aves.
II. Trecho médio: maior densidade populacional, ainda com características de áreas rurais, mas não tão predominante como na parte superior.
III. Trecho inferior: representado por um grande volume populacional e industrial

Segundo o censo demográfico, dos municípios inclusos nesse estudo de 2010, as maiores taxas da população residente em áreas rurais pertencem ao trecho superior, corroborando com dados fornecidos pela FEPAM (2014). No entanto, no município de Nova Santa Rita, o qual pertence ao trecho inferior, se observa uma maior taxa de indivíduos que residem em áreas rurais comparado com os municípios de Igrejinha, Sapiranga e Campo Bom, pertencentes ao trecho médio (Figura 1) (IBGEa, 2010). Sendo assim, a parte inferior da bacia não é composta apenas por áreas urbanas e industriais, como já demostrado também no Balanço Hídrico da BHRS publicado em 2015 (Figura 2), que avaliou as disponibilidades e demandas por água na bacia dos Sinos e, mostrou que no trecho inferior do Vale do Rio dos Sinos é onde se tem maior demanda de água para fins agrícolas. Sendo, de um total de 3,81m3/s água, 85% é destinada para irrigação de arroz no trecho baixo, 13% para os municípios do trecho alto e apenas 2% vai para o trecho médio (COMITESINOS, 2015).

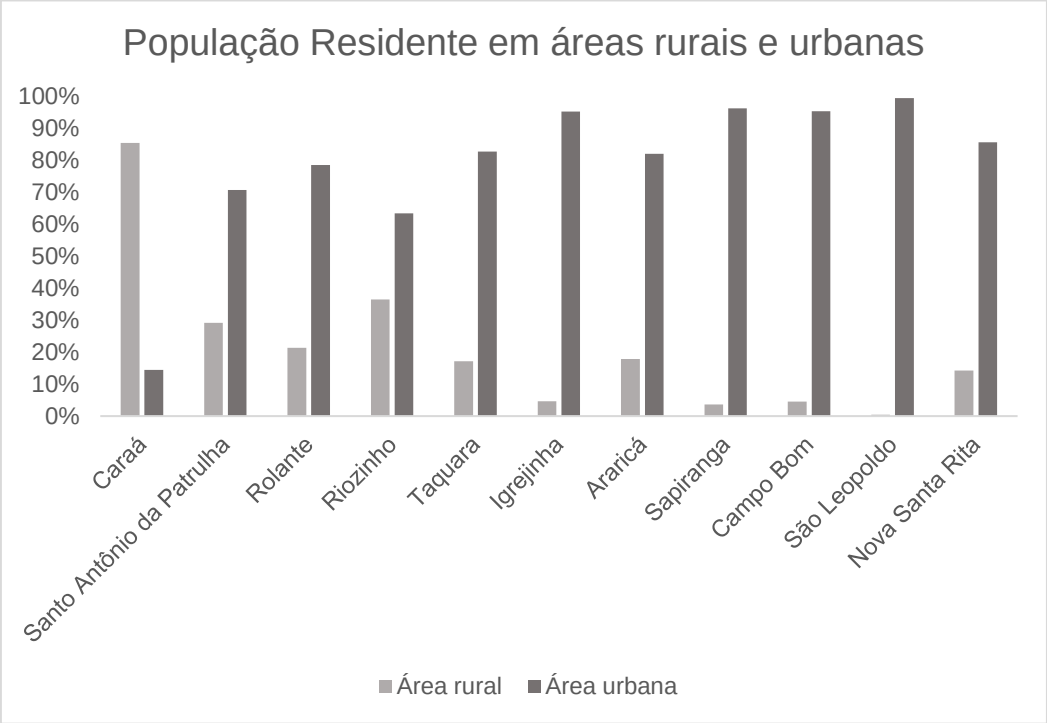

Figura 1: Percentuais de população residente em áreas rurais e urbanas dos municípios incluídos no estudo. Municipios pertencentes ao trecho superior: Caraá e, Santo Antônio da Patrulha; trecho médio: Riozinho, Rolante, Sapiranga, Araricá, Taquara, Igrejinha e, Campo Bom; trecho baixo: Nova Santa Rita e, São Leopoldo.

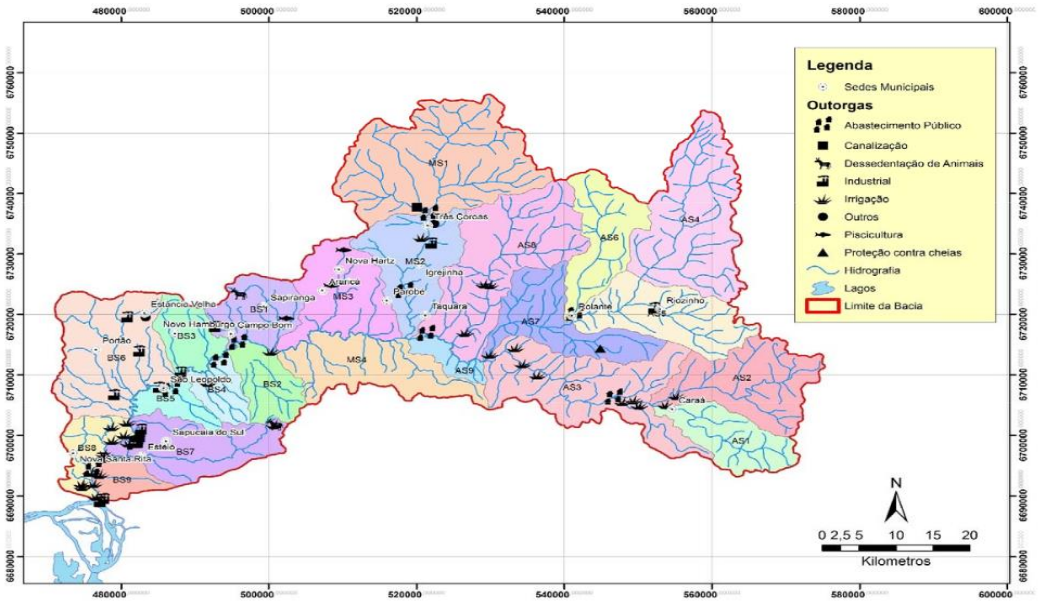

Figura 2: Balanço Hídrico da BHRS

1.6 Saneamento e acesso a água em áreas rurais

O acesso universal ao saneamento adequado é fundamental e um direito de todos. No entanto, ainda no ano de 2015, 2,4 bilhões de pessoas no mundo não dispõem de instalações sanitárias melhoradas, como banheiros de descarga ou latrinas de laje. Entre estas, 950 milhões ainda praticam defecação aberta. Contudo, essa disparidade é acentuada nas populações mais pobres e aqueles que vivem em áreas rurais (UNICEF, 2016). O acesso ao saneamento básico, assim como à água potável, contribui muito para redução de doenças e mortes, principalmente entre as crianças. Entretanto, esses serviços atendem principalmente as zonas urbanas. (WHO e UNICEF, 2015; UNICEF, 2016). No mundo, a cobertura de água potável em zonas rurais de países em desenvolvimento é de 84 %, enquanto nas áreas urbanas a cobertura desses serviços pode chegar a 96 % (WHO / UNICEF, 2015).

Não obstante, no Brasil, de acordo com o censo demográfico de 2010, o percentual de domicílios atendidos por redes de abastecimento de água potável é de 92 % em áreas urbanas, contra apenas 28 % em áreas rurais (IBGEb, 2010). Esse elevado contingente de domicílios à margem da distribuição de água tratada, faz com que a população que reside nessas localidades dependa de outras fontes, como o uso de vertentes, poços artesianos e/ou cavados. No entanto, essas fontes são muitas vezes de qualidade duvidosa, não recebem tratamento e, nem são monitoradas rotineiramente, colocando em risco a saúde de animais e humanos que usufruem dessas águas (Perdomo et al., 2013).

Devido esse déficit nos serviços de saneamento básico em áreas rurais, é ainda comum o descarte direto de dejetos em arroios por canalização rudimentar, sendo raro o uso de fossas sépticas nos domicílios (Spilki et al., 2013). Quanto aos dejetos animais, não é ainda amplamente adotado o uso de esterqueiras ou de biodigestores, sendo habitual a utilização de aterros e aplicação de dejetos de efluentes direto no solo e nas fontes hídricas (Do Amaral, 2004; De Oliveira et al., 2012). Em consequência da disposição inadequada de resíduos orgânicos e inorgânicos gerados pela agropecuária, bem como a falta de instalações sanitárias. Essas regiões tornam-se mais propícias a degradação ambiental e a contaminação da água. (De Oliveira et al., 2012; Spilki et al., 2013)

1. OBJETIVO GERAL

Expandir o conhecimento sobre a contaminação de diferentes fontes de águas no contexto zona rural de municípios do Vale do Rio do Sinos, através da detecção de *E. coli* e diferentes espécies de AdV, avaliar a infecciosidade e comparar as técnicas moleculares empregadas para detecção de HAdV.

2. OBJETIVOS ESPECÍFICOS

I. Detectar e quantificar genomas de HAdV, CAV, BAdV, PAdV e AvAdV através qPCR em amostras de águas subterrâneas e superficiais;
II. Avaliar a diversidade de AdV através de métodos de sequenciamento genético e análise filogenética molecular;
III. Analisar a infecciosidade dos HAdV nas amostras utilizando por ICC-qPCR
IV. Comparar as técnicas moleculares utilizadas para a detecção de HAdVS com as principais demandas de usos múltiplos da água e do solo por trecho da bacia.

3. ARTIGO

Esse artigo foi submetido ao periódico Water Research.

MICROBIAL SOURCE TRACKING IN SMALL FARMS BASED ON DIFFERENT METHODS EMPLOYED FOR ADENOVIRUS DETECTION

Meriane Demoliner[a*]; Juliana Schons Gularte[b]; Viviane Girardi[c]; Ana Karolina Antunes Eisen[d]; Fernanda Gil de Souza[e]; Rodrigo Staggemeier[f]; Andreia Henzel[g]; Fernando Rosado Spilki[h]

Laboratório de Microbiologia Molecular, Universidade Feevale, ERS 239, 2755, Vila Nova, Novo Hamburgo, 93352-000, Brazil

* Corresponding author: Laboratório de Microbiologia Molecular, Universidade Feevale, ERS 239, 2755, Vila Nova, Novo Hamburgo, 93352-000, Brazil. Electronic address: merianedemoliner@gmail.com

ABSTRACT

This study aims at the expanded knowledge about the fecal contamination by humans and animals, using the Adenovirus (AdV) as bioindicators in different water sources from rural areas, also evaluating the infectivity and comparing the different techniques used to detect the *Human mastadenovirus* (HAdV). To that, it was collected 124 samples, being 86 from groundwater and 38 of surface water. In November and December of 2015 the samples were collected, in 34 rural properties of 11 municipalities located along the Rio dos Sinos basin. Escherichia coli count was realized by Colilert kit and, the samples were submitted the detection and characterization tests of AdV by different methods (qPCR, multiplex-qPCR and, nested-PCR). In addition, the viral infectivity was realized too by ICC-qPCR. *E. coli* was detected in 62.8% of groundwater samples, with the geometric mean of 16.7 MPN/100mL and, in surface waters the detection rate was 68.4%, with the geometric mean of 5.08x102 MPN/100. Amongst the viral indicator in the groundwater, the HAdV was detected in 48.84% of samples, followed by Canine mastadenovirus (CAV) 19.77%, Bovine mastadenovirus (BAdV) 17.44%, Aviadenovirus (AvAdV) 15.12%, and Porcine mastadenovirus (PAdV) 3.49%. In surface water was found the following results: HAdV 44.74%, CAV 42.11%, BAdV 28.95% and, PAdV and AvAdV, both were found in 13.16%. The quantification of genomic copies per liter ranged from 9.40 x104 to 5.54x1010cg/L. In groundwater samples, it was possible to observe 11.6% infectious samples, and in surface water18.4%. The results showed a increased in the sensitivity and, consequently the number of positive samples when were used a set of techniques for HAdV detection.The study shows that the environment studied suffer on anthropic action, which the lack of basic sanitation facilities is an important forerunner for the fecal pollution found in the waters evaluated in that research.

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Keywords: Adenovirus. Bioindicator. Fecal contamination. Groundwater. Surface water. Rural area.

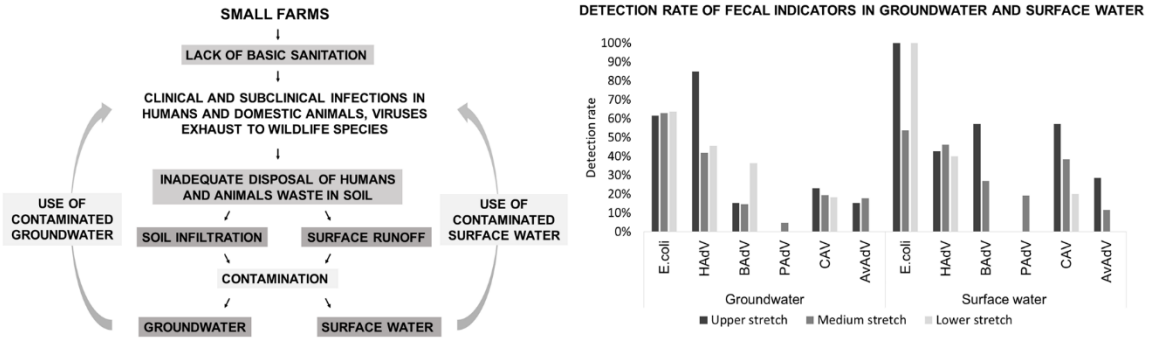
HIGHLIGHTS

HAdV is a reliable viral indicator with higher detection rate in groundwater and surface water in farms

Using only one diagnostic tool to detect viruses in environment may not be sufficient

The HAdV-C isolated in cell culture had not had been detected directly by any molecular detection technique

GRAPHICAL ABSTRACTS



4.1 INTRODUCTION

The universal access to basic sanitation is a fundamental right of all. Though, still in the year 2005, 2.4 billion people still do not have basic sanitation facilities. Among these, nearly 10 million people still practicing open defecation. However, such disparities are accentuated in the poorest populations and those living in rural areas (WHO/UNICEF, 2015). The access to basic sanitation as well as drinking water contribute significantly to disease and death reduction, mainly among children (WHO/UNICEF, 2015; WHO, 2017). Because the provision of drinking water was one of the measures more successful by humanity in terms of disease control and prevention (Ashbolt, 2015). Globally, in rural areas, the drinking water cover is of 84%, While in urban areas the cover of such services can reach to 96% (WHO/UNICEF, 2015). In Brazil, acording with the demographic census of 2010, 93% of households in urban areas receive drinking water against only 29% those in rural areas (IBGE, 2010).

However, this high contingent of households on the margins of the distribution of treated water, it makes the population living at these locations dependent on other water sources, as well as water springs, artesian wells or shallow wells. Usualy these water sources are of doubtful quality, they do not receive adequate treatment and not even are routinely monitored, representing a danger the animal and human health that enjoy these waters (Perdomo et al., 2013).

Enteric viruses are microorganisms associated with environmental contamination and infections caused by consumption of water and food contaminated (Bosch et al., 2008). These pathogens are eliminated in large quantities in the feces of humans and animals and they have an easy of dispersed into the environment, so these viruses are frequently found in river, groundwater, recreation water, drinking water, wastewater and sewerage (Bosch et al., 2008; Staggemeier et al., 2017). Among those, stand out the viruses of *Adenoviridae* family, that is composed of five genera: *Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus* and *Siadenovirus* (ICTV, 2016). Adenoviruses (AdV) It is a nonenveloped virus containing dsDNA genome and with an icosahedral nucleocapsid, it is the approximate size of 60 a 100 nm diameters and has trait projections (Santos, Romanos and Wigg, 2015).

Human mastadenovirus(HAdV) belong to the *Mastadenovirus* genus which encompasses 86 genotypes are sub-divided into seven species of A until G (ICTV, 2016; HADVWG, 2018). HAdV are able to infect different tissues and systems that allow different types of infections, however, the intestine and the tract respiratory still are the main sites for replication for these viruses (Santos, Romanos & Wigg, 2015). HAdV is an important etiologic agent of diarrhoeal disease, is considered the second most important viral pathogens causing child gastroenteritis only losing for the rotavirus (Fong et al., 2010). The HAdV of species F are commonly reported as causing of Infections in the gastrointestinal tract. However other species also are associated with that type of illness, as the HAdV-A, B, D e G (Santos, Romanos & Wigg, 2015; Rames et al., 2016). Whereas, the HAdV of species C are responsible for causing respiratory diseases. These viruses can replicate in the respiratory tract and to be eliminated by intestine without any diarrhea signal. The infection by HAdV-C is one of the most common causes of upper respiratory tract infections in children (Roy et al., 2009). In some individuals, these infections that can develop complications as respiratory failure, intractable convulsions as cyanosis, tachycardia, and hypotension (Cheng et al.,

2015). While the HAdV-B and E are more associated with serious acute respiratory infections. Even that the majority of infections by HAdV in immunocompetent host occurs asymptomatically way, in immunocompromised individuals, children and elderly, it can result in high morbidity and mortality rates. Due to the increase in the number of transplantation patients and patients with human immunodeficiency virus (HIV), the severe infections caused by HAdV it has been each time more frequent (Lenaerts et al., 2005).

Besides human beings, species-specific AdVs can also infect a wide range of animal species, including mammals, birds, reptiles, amphibians and fish (Benkő et al., 2002). *Canine mastadenovirus* (CAV) belong to the *Mastadenovirus* genus and the A species (ICTV¸ 2016), that is divided into two genotypes (CAV-1 e CAV-2), whom the CAV-1 induced a systemic disease that have how the main complication the infectious canine hepatitis, today a rare clinical sign, but usually severe. On the other hand, the CAV-2 replicate mainly in respiratory epithelium, can cause tracheobronchitis characteristic of the kennel cough complex in association with the canine parainfluenza virus type 2 and *Bordetella* sp. As well as of domestic dogs the infections by CAV can occurring in other species how coyotes, red foxes, and wolves. In these wild species, the infection can be fatal (De Almeida Curi et al., 2010; Dowgier et al., 2016). The *Porcine mastadenovirus* (PAdV) are part of the *Mastadenovirus* genus and are divided into three species PAdV-A, PAdV-B e PAdV-C (ICTV, 2016), normally they no cause serious illnesses although there are reports of cases of pneumoenteritis and encephalitis (De Motes et al., 2004). The *Bovine mastadenovirus* (BAdV) belong of two distinct genera, whon the BAdV-A to BAdV-B and BAV-C species belonging to the genus *Mastadenovirus* and BAdV-D species to the genus *Atadenovirus* (ICTV, 2016). Some genotypes of BAdV are associated with clinical scenario of keratoconjunctivitis, acute febrile disease or pneumoenteritis and in young animals, the no proper management colostrum-deprived can be fatal (De Motes et al., 2004). The *Aviadenovirus* (AvAdV) belong to the *Aviadenovirus* genus and are divided in 7 species, all responsible for infect only birds. The AvAdV causing mainly respiratory and digestive infections, how bronchitis, enteritis and diarrhea and some species can be presented hepatitis, hepatomegaly, and haemorrhages (Moraes & Costa, 2017).

As these pathogens are ubiquitous in the environment, studies have been shown the great importance of research and insertion of fecal indicators of viral origin

for the water pollution assessment (Hewitt et al., 2013; Rames et al., 2016). Different species of enteric viruses can be found in aquatic environments that are in accordance with standards of bacterial indicators (Dalla Vecchia et al., 2015; Fong & Lipp, 2005). The viruses are host specific which allows a better characterization of contamination font different of *Escherichia coli* (*E. coli)* that is a bacterium invariable found in human and animals excrements (Fong, Griffin & Lipp, 2005; Gotkowska-Płachta et al., 2015). Within the group of enteric viruses, the AdVs stands out due to the structural characteristics that give your greater stability in adverse conditions, including temperature and pH changes, physical and chemical treatments as filtration and chlorination processes, these methods more employees in water treatment currently (Fong & Lipp, 2005; Thurston-Enriquez et al., 2003; Barardi et al., 2012). Also, the AdVs tend to be more resistant to ultraviolet radiation (UV) when compared with RNA viruses, considering that the HAdV can use by host cell enzymes to repair your DNA damage. Thus, allowing for them a greater persistence in staying infectious in environment by a long period (Thurston-Enriquez et al., 2003; Ko, Cromean & Sobsey, 2005).

This research aimed through different tools analyze the microbiology quality of groundwater and surface water in rural areas. In addition to using the traditional parameters as the *E. coli*, that study propose to investigate virus of different species in order to can to characterize the main font of pollution. Moreover, it searches to contribute the use of different methods that be use in enteric viruses analyses in environment samples due that exist many studies that use different methods for HAdV detection, but when it comes of detection that microorganism in water, little is known about the sensibility and specificity.

4.2 MATERIAL END METHODS

4.2.1 Sampling

The Rio dos Sinos basin (*Bacia Hidrográfica do Rio dos Sinos*- BHRS), is located to the northeast of the State of Rio Grande do Sul (RS), Brazil. It covers 32 municipalities and supplies about 1.5 million people. The basin is currently divided into 3 distinct sub-stretches, being: a) upper stretch: represented by areas of small rural

properties, with low population density, where agriculture and small dairy farms, pigs and poultry predominate; b) medium stretch: higher population density, still with characteristics of rural areas, but not as predominant as in the upper part; c) lower stretch: represented by a industrial and population large volume (Dalla Vecchia et al., 2015; FEPAM, 2014).

However as demonstrated in the SRB water balance published in 2015, which evaluated the availability and demand for water in the Sinos basin. Showed that in the lower reaches of the Rio Sinos Valley are where there is a greater demand for water for agricultural purposes. That of a total of 3.81m3/s water, 85% is destined for irrigation of rice in the low stretch, 13% for the municipalities of the high stretch and only 2% goes to the middle stretch (COMITESINOS, 2015). Thus, showing that the lower part of the basin is not only composed of urban and industrial areas.

The collections were carried out on November and December of 2015, in 34 rural properties of 11 municipalities located along the BHRS (Figure 1). Water samples (500 mL) were collected in sterile bottles, 86 from groundwater (spring and artesian well) and 38 of surface water (streams, weir and river), totalizing he 124 samples.

This research received the support of RS State Technical Assistance and Rural Extension Company (*Empresa de Assistência Técnica e Extensão Rural do Governo do RS* - EMATER/RS), that is an institution that has the goal to promote sustainable rural development in the State of RS. Therefore, the rural proprieties that were select, are locals which that institution has been carrying works, thus, it was not possible to choose the propriety where was realized the collects for the best distribution them in the study region.

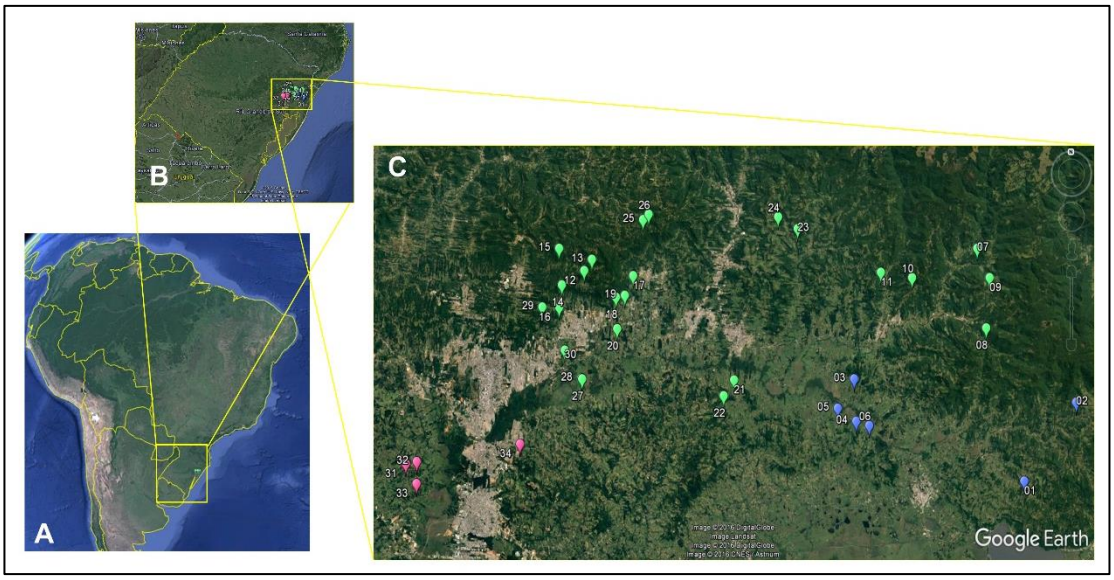


Figure 1. A) Brazil in South America B) Map of Rio Grande do Sul in Brazil. C) Distribution of rural properties in the Vale dos Sinos region. In blue the upper section: property 01 and 02 belonging to Caraá; 03 to 06 Santo Antônio da Patrulha. In green the middle stretch: 07 to 09 referring to Riozinho; 10 e11 Rolante; 12 to 16 Sapiranga; 17 to 20 Araricá; 21 to 23 Taquara; 24 to 26 Igrejinha; 27 to 30 Campo Bom. And in pink the lower section: from 31 to 33 of Nova Santa Rita and 34 from São Leopoldo.

4.2.2 *Escherichia coli* detection and quantification

The detection and quantitation of *E. coli* were performed by Colilert® (IDDEX) kit, following the manufacturer's instructions. In the cases that the most probable number (MPN) of bacteria exceeded the method's detection limit, the samples were diluted within 24 hours after collection and the technique was applied again.

4.2.3 Viral concentration by ultracentrifugation and nucleic acid extraction

All water samples were concentrated by ultracentrifugation method (Girardi et al., 2018). Aliquots of 36 mL were centrifuged at (Sigma® 3-30KS equipment–Germany, rotor 12150-H) for 3 h at 41,000 × g, at 8 °C. After this step, the precipitates were resuspended and vigorously homogenized in the vortex for 1 min with 1 mL of Tris-EDTA buffer (pH 8.0). The DNA of concentrated samples were extracted by BioPur® kit, following the instructions the manufacturer's instructions. The final elution (60 µL) was performed in microtube nuclease-free.

4.2.4 Detection and quantification by real-time polymerase chain reaction (qPCR) and multiplex-qPCR

The qPCR was performed to two different reactions: First, using the VTB1 oligonucleotides pair, targeting the HAdV-F and, second using the VTB2 oligonucleotides pair targeting the HAdV-C. The multiplex-qPCR was performed using the AdV oligonucleotides pair with HAdV, CAV, BAdV, PAdV, and AvAdV targets. All reactions can detect and quantify the sequences target (Table 1). To a final reaction volume of 25 µL, 2.5 µL of SYR® Green supermix (Invitrogen ™ Platinum® SYBR® Green qPCR SuperMix-UDG), 5.5 µL nuclease-free water, 1 µL of each oligonucleotides pair, and 5 µL of extracted DNA ware used. The qPCR assays were performed at iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) with iQ™5 optical system software 2.1 version. Being the sensitivity of the reactions 6.2×10^{1} gc/5 µL (Dalla Vecchia et al., 2015). The assays were performed in 96-well plates, and, all samples were tested in duplicate, including negative controls, and a standard five-point curve formed by serial dilution of positive control with known quantification. Where required, the samples were diluted 1:10 (DNA) in nuclease-free water, in order to reduce the PCR inhibitory substances present in the samples. The conditions of the qPCR for the oligonucleotides VTB1 and VTB2 were an initial incubation of 2 min at 50 °C, 10min at 95 °C, 45 cycles of 20 s at 95 °C for denaturation, and 1 min at 55 °C for annealing. After this step, a denaturing curve was made to check the specificity of amplification products (melting step between 55 and 95 °C). For the AdV oligonucleotides pair, the qPCR conditions were the same, however, the annealing temperature was 58 °C.

Table 1. Oligonucleotide used to perform the qPCR and multiplex-qPCR

Target	Oligonucleotides name	Sequencies 5'→3'	Position	Product length (bp[1])	Melting curve by species (°C)
*HAdV-F (HAdV 40, 41) Hexon gene	VTB1-HAdVFf VTB1-HAdVFr	GCCTGGGGAACAAGTTCAGA GCGTAAAGCGCACTTTGTAAG	336-355 453-473	137	86.5
*HAdV-C (HAdV 1, 2, 5, 6) Hexon gene	VTB2-HAdVCf VTB2-HAdVCr	GAGACGTACTTCAGCCTGAAT GATGAACCGCAGCGTCAA	106-126 190-207	101	86.5
**HadV; CAV 1–2; BAdV; PAdV; AvAdV Hexon gene	ADV-F1 ADV-R1	CAGTGGTCGTACATGCACAT TCGGTGGTGACGTCGTGG	4–23 67–86	130	HAdV- 88; CAV − 82; BAdV−85,5; PAdV−83.5; AvAdV−80,5.

* WOLF et al. (2010) used in two different reactions of qPCR
** LUZ et al. (2015) used in a reaction of multiplex-qPCR
[1] base pair

4.2.5 Nested polymerase chain reaction (Nested-PCR)

The Nested-PCR was performed to detect the presence of the AdV genome of different hosts. To a final volume of 50 µL were used 25 µL of (GoMaq® Master Mix colorless - PROMEGA), 18 µL nuclease-free water, 1 µL of each oligonucleotide (Pol-F 5'-CAGCCKCKGTTRTGYAGGGT-3' and Pol-R 5'- GCHACCATYAGCTCCAACTC-3'), and 5 µL of extracted DNA. The assay conditions were an initial incubation step at 94 °C for 5 min, 40 cycles of amplification, consisting of denaturation for 30 s at 94 °C, annealing at 50 °C for 30 s (-0.5 °C per cycle), and extension at 72 °C for 1 min, and a final extension stage at 72 °C for 10 min. The second PCR reaction was performed using the same reagents and quantities from the first step, as well as the amplification cycles. But, just replacing the oligonucleotide pair, using in the second the Pol-nF 5'-GGGCTCRTTRGTCCAGCA-3' and Pol-nR 5'-TAYGACATCTGYGGCATGTA-3', and the 5 µL of extracted DNA was replaced by first PCR products (Li et al., 2010). At the end of the second amplification, that products were subjected to 2% agarose gel electrophoresis with 0.5 mg of ethidium bromide/mL. And the molecular sizes of the products were compared to a DNA standard of 100 bp (Ludwig). After those amplicons were visualized, they were purified using a QiaQuick DNA purification kit (Qiagen)and sent to sequencing.

4.2.6 Genetic sequencing and phylogenetic analysis

The DNA sequencing of amplicons obtained from nested-PCR was performed by the Sanger method. To identify the DNA fragments, nucleotide sequences were assembled using the CAP3 computer program implemented in the BioEdit 7.0.5 suite. that way, the sequences obtained were compared with other nucleotide fragments available from GenBank, according to the Neighbor-Joining methodology (Saitou & Nei, 1987). Followed the phylogenetic tree was elaborated from the calculation of evolutionary distances, using the Kimura-parameter 2 method (Kimura, 1980) and operating with Molecular Evolutionary Genetics Analysis version 5 (MEGA5) software (Tamura et al., 2007).

4.2.7 Detection of HAdV viability by integrated cell culture quantitative PCR (ICC-qPCR)

To detect the presence of infectious viruses in the samples, the A549 cells line (human lung carcinoma) was used based on its permission to HAdV, such cells were maintained with Eagle's minimal essential medium (E-MEM), supplemented with 10 % fetal bovine serum (FBS), and 1% Penicillin-Streptomycin (10,000 IU/mL-10 mg/mL). Cells were cultured in 24-well plates and after 24 h approximately the supernatant E-MEM was removed and replaced with 200 µL of the concentrated water samples, previously diluted (1:2) with E-MEM and filtered with a membrane of 0.22 µm. The plates were kept in incubators for 2 h with uniform stirring every 15 min. After this incubation period, the inoculum was removed and 1mL of the cell maintenance medium was added, but without FBS. Plates were maintained in incubators at 37 °C with 5 % CO_2 atmosphere for five days and after this period, the plates were frozen at -80 °C. To the second passage in cells, the plate from the first passage was thawed three times and the previous process procedure was repeated, except the filtration step because in this time, the inoculum used was 200 µL the result of the first passage. That process was repeated until the third passage. To assess if there were integral particles, the first and third pass samples were treated with DNAse. Subsequently, the same samples were submitted to extraction of nucleic acids, and to qPCR using the oligonucleotide pair VTB1 and VTB2 for the detection of HAdV genomes.

4.2.8 Comparison of techniques of HAdV detection

A Venn diagram was elabored in order to obtain the best view of differents results obtained with three different tests, both tests are able to detect the HAdV. To that, only the results from that used the AdV, VTB2, DNApol oligonucleotides were considered.

4.3 RESULTS

4.3.1 *E. coli*

The presence of *E. coli* was detected in 62.8 % (54/86) of the groundwater samples, ranging from 1 to $1.99x10^3$ MPN/100 mL (geometric mean = 16.7 MPN/100 mL). In surface water *E. coli* was detected in 68.4 % (26/38) of the samples with the counting ranged from 5 a $4.35x10^4$ MPN/100 mL (geometric mean $5.08x10^2$ MPN/100 mL).

4.3.2 Viral analyses in the groundwater and surface water samples

In the groundwater the HAdV was viral indicator with higher detection rate, it was in 48.84% (42/86) of the samples. In that positives samples the minimum quantification was 9.40 $x10^4$ genomic copies per liter (gc/L) and the maximum was $4.51x10^{10}$ gc/L, followed respectively by CAV 19.77 % (17/86; $5.85x10^7$ ─ $5.54x10^{10}$ gc/L), AvAdV 15.12 % (13/86; $9.07x10^6$ ─ $6.52x10^8$ gc/L), BAdV 17.44 % (15/86; $7.02x10^6$ ─ $3.57x10^9$ gc/L) e PAdV 3.49 (3/86; $9.07x10^6$ ─ $2.92x10^8$ gc/L) (Figure 2). In the surface water samples, the HAdV was presented with highest occurrence too, it was detected in 44.74 % (17/38) whit quantification between $5.81x10^5$ to $1.55x10^{10}$ gc/L,followed of CAV 42.11 % (16/38; $4.13x10^7$ ─ $1.55x10^{10}$ gc/L), BAdV 28.95 % (11/38; $1.65x10^7$ ─ $1.55x10^{10}$ gc/L), and finally, with less detection rate PAdV and AvAdV, both were found in 13.16 % (5/38; 2.8510^8 ─ $2.74x10^9$ gc/L e $7.38x10^7$ ─ $9.77x10^9$ gc/L, respectively) (Figure 2). At the figure 3, it is possible to observe the different detection rate in groundwater and surface of the viral indicators comparing them with *E. coli* in different stretches of the Rio dos Sinos basin.

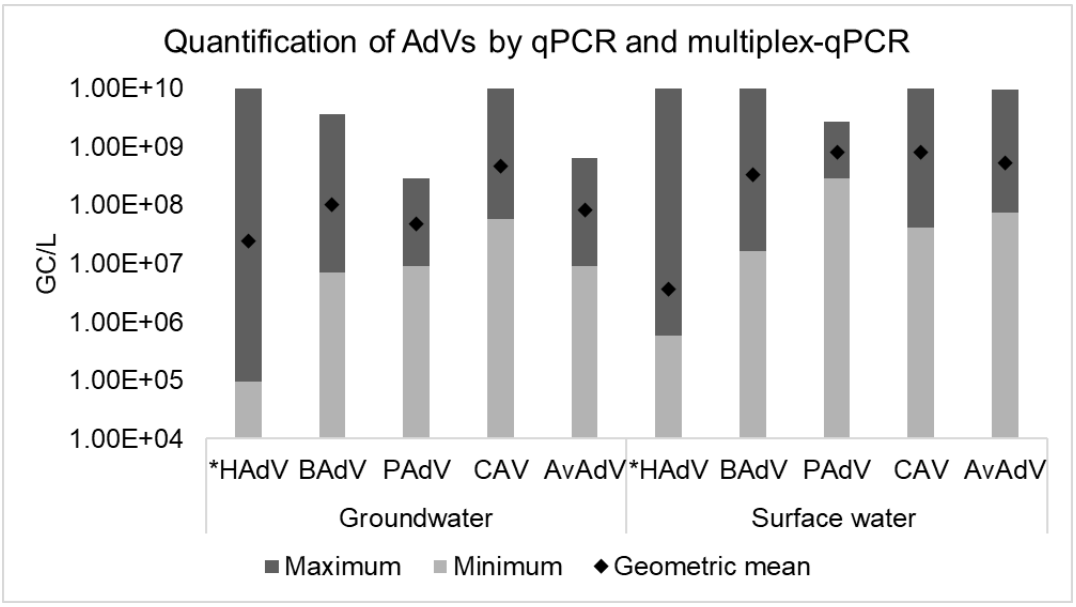


Figure 2. Maximum, minimum and geometric mean value of genomic copies per liter water (gc/L) of the HAdV, BAdV, PAdV, CAV and AvAdV, that were detected and quantified by multiplex-qPCR using AdV oligonucleotides in groundwater and surface water samples. * To HAdV, besides the results obtained at multiplex-qPCR, here have been considered too, the results from qPCR using VTB1 and VTB2 oligonucleotides.

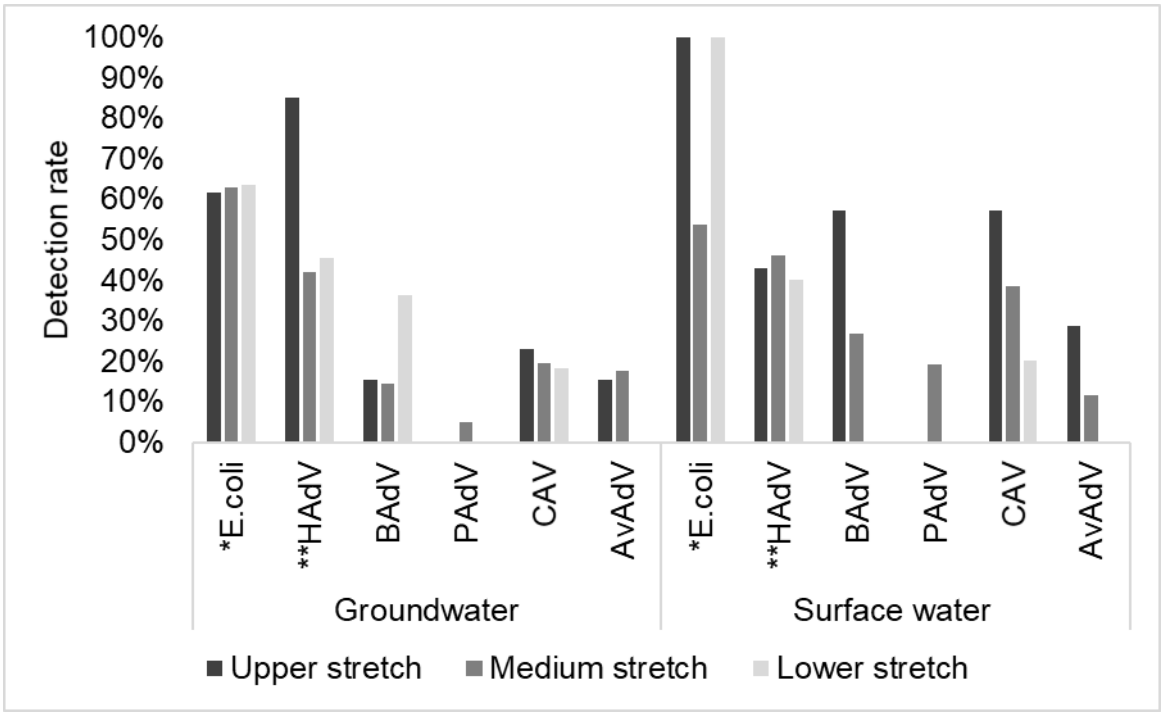


Figure 3. Detection rate in groundwater and surface of the indicators in different stretches of the Rio dos Sinos basin. BAdV, PAdV, CAV and AvAdV were detected by multiplex-qPCR using AdV oligonucleotides. **E. coli* detection was perfomed by Colilert kit. **To HAdV, besides the results obtained at multiplex-qPCR, here have been considered too, the results from qPCR using VTB1 and VTB2 oligonucleotides.

4.3.3 Phylogenetic analysis

The phylogenetic analysis demonstrated that were found three different species of AdV (HAdV-C, HAdV-F e and BAdV), both belonging to the *Mastadenovirus* genus, being the C species the most predominant (Figure 4).

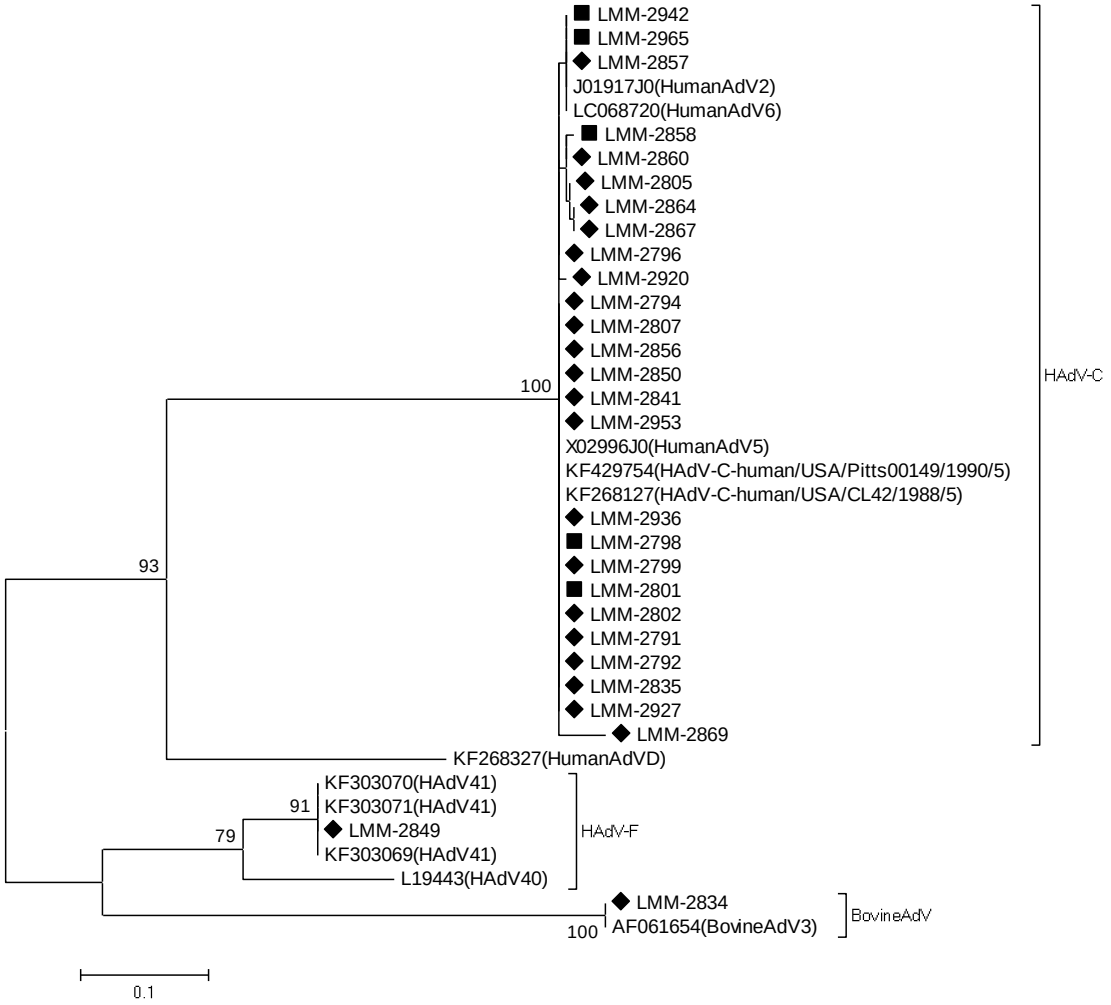


Figure 4. Phylogenetic analysis of AdVs. Neighbor-joining tree constructed using AdV sequences obtained by Sanger sequencing, and reference strains from the NCBI GenBank database. The samples analyzed in this study are marked with a diamond for groundwater and a square for surface water. Bootstrap values are indicated at each tree root.

4.3.4 Viral infectivity analysis

In groundwater samples, it was possible to observe 10/86 infectious samples, being five in the first passage in cells, and six in the third passage. However, only one sample of the first passage remained infectious too in the third passage. In surface

water, the total of infectious samples were 07/38 (18.4%), but only two samples were infectious in the first passage and five in the third. In this samples none samples of the first passage remained infectious in the third passage (Table 2). There were infectious samples only for the HAdV-C, none sample was infectious for HAdV-F, and infectivity tests for animal viruses were not performed. Four of the samples that were infectious for HAdV-C had not detected the HAdV genome by any of the oligonucleotides that detected viruses of human origin. As well as five of the surface water samples, which were only able to detect the HAdV-C genome after passage of the samples into cells. In addition, of 17 infectious samples, only one was possible to be isolated, namely isolate LMM 2863(HAdV-C). Probably the HAdV was below the detection limit of qPCR and only be detected after at third passage with $3.81x10^2$ gc/5µL and at tenth passage the quantification was $1.35x10^4$. The isolate LMM 2836 not provide any cytopathic effect despite being detected by molecular techniques.

Table 2. Infectivity analysis of HAdV-C quantification in gc/5µL by qPCR using the VTB2 oligonucleotides pair.

		Cell passages				Cell passages	
	Samples	#1	#3		Samples	#1	#3
Groundwater	LMM 2799	$1.29x10^4$		Surface water	LMM 2798	$1.27x10^2$	
	LMM 2805		$1.84x10^2$		LMM 2804		$1.90x10^2$
	LMM 2819	$6.81x10^2$			LMM 2830		$2.43x10^2$
	LMM 2862	$9.06x10^2$	$4.40x10^2$		LMM 2871		$3.71x10^2$
	LMM 2864		$3.81x10^2$		LMM 2832	$8.67x10^3$	
	LMM 2865	$2.83x10^3$			LMM 2863		$3.81x10^2$
	LMM 2866		$3.54x10^2$		LMM 2959		$3.79x10^2$
	LMM 2953		$1.29 x10^4$				
	LMM 2967	$4.15x10^3$					
	LMM 2968		$1.66 x10^3$				

#1 represents the first passage in cells
#3 represents the third passage in cells

4.3.5 Comparison of techniques of HAdV detection

The reaction that obtained the highest number of positive samples for the HAdV was that the one used the AdV oligonucleotides pairs, totaling 32 (26%) positive samples. Followed by reaction with DNApol 26 (22%) and, last but the reaction that used VTB2 pair and detected HAdV in 21 (17%) samples. Analyzing individually each reaction, the differences among them do not seem so big, but considering the three reactions, the number of positive samples is 58 (47%). This is because within the three

reactions that can detection of HAdV genome, 41 samples were positive individually just in a reaction, 12 in two reactions and, only four samples were positives in three reactions, as demonstrated at figure 5. In addition, it was observed HAdV-C infectivity in 19 (15%). However, in nine samples the HAdV genome were only detected after they had been passed in cell culture. Therefore, out of a total of 124 samples, 67 (54%) were HAdV positives. In this comparison, the VTB1 oligonucleotides pair were not considered because for this essay only one sample was positive.

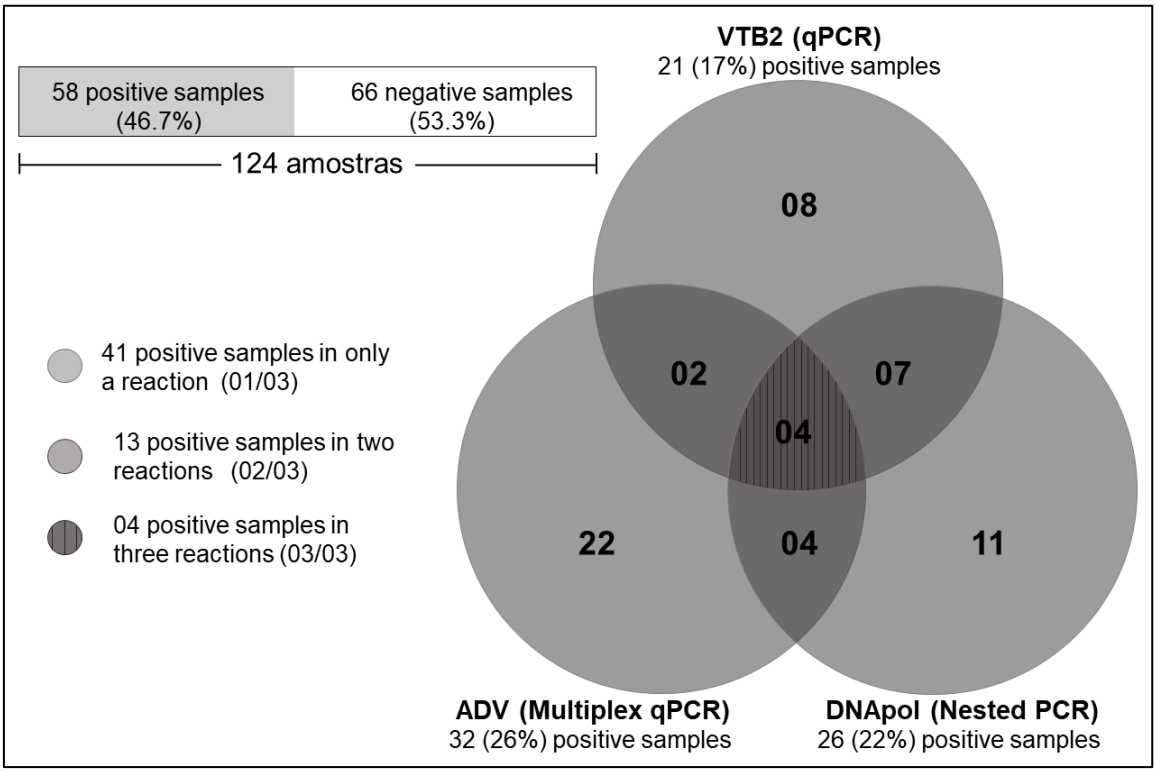


Figure 5. The left image shows the percentage and number of positive samples for one or more of the three oligonucleotides tested. And the right the Venn diagram show the combinations between reactions

4.4 DISCUSSION

In most countries in the world, only bacterial indicators has been used to monitor the quality of drinking and recreational water. In Brazil, the National Council for the Environment (*Conselho Nacional do Meio Ambiente* - CONAMA), the responsible of the classification of water bodies. However, only thermotolerant coliforms as microbiological criterion are used to establish these classifications. Enabling the use

of *E. coli* as a substitute indicator provided that meets the limits established by the competent environmental agency (CONAMA, 2005; CONAMA, 2008). In the United States, the US Environmental Protection Agency (EPA), uses thermotolerant coliforms as indicator to evaluate the contamination of water, *E. coli* for freshwater and enterococci for marine and salty waters (EPA, 2010). Moreover, European and National Drinking Water Quality Standards does not include viral indicators in terms microbiological standards (NIEA, 2011). However, the present study show us that the use of only bacterial indicators for water evaluation is insufficient, as has already been shown in groundwater of the 32 negative samples, only 11 (34 %) were also negative for viral contamination indicators, while 21 (66 %) of these negative samples for *E. coli* were positive for one or more viral indicators. In surface waters, all samples (12) that were negative for *E. coli* were contaminated with some type of viral indicator. Corroborating with prior studies have showed that there is no correlation between the presence of *E.coli* and enteric viruses (Dalla Vecchia et al., 2015; Peteffi et al., 2018; Skraber et al., 2004).

The HAdV was the viral indicator most abundant, it presented a mean rate of detection in surface and groundwater of 43%, with exception of upper stretch's groundwater, which the HAdV was detected in 85% of samples. As mentioned by Spilki et al. (2012) is expected lower levels of contamination in areas that have the lowest population density, as is a case of the basin upper stretch, however, the great impact of poor sanitation in this region can overcome this. That situation already observed in another study, that that in assessing groundwater from upper stretch's BHRS, obtained a detection rate of 83% to HAdV (Staggemeier et al., 2015). By virtue of deficiency of basic sanitation services in rural areas, is observe yet the direct disposal of waste in streams by rudimentary channelling and the use of household septic tank is rare yet (Spilki et al., 2013). These facts explain the high detection rate found in the study's regions and justify the high concentration of genomic copies that was detected, in this study the geometric mean is like the concentration of wastewater and sewage samples from developed countries (Dong, Kim & Lewis, 2010; Fong et al., 2010). However, in Brazil it was not the first time that these concentrations were observed in surface water. In Rio de Janeiro city, the concentration of HAdV-C was up to 10^9 gc/L (Staggemeier et al., 2017) and in surface water streams from BHRS region already was detected concentration up to 3.28×10^8gc/L (Peteffi et al., 2018).

Among the viral indicators from animals origins, the CAV was with the greatest detection rate. Which can be explained by the high presence of domestic animals, in which, especially dogs have been considered a reservoir for many infectious agents and believe that they are more affected than wild animals (Fiorello et al., 2004). The dogs are much associated with the human presence and are usually found in high number in rural properties. However, the proximity of them with the natural habitat of wild species may disturb them. Given the great detection rate of CAV found in surface water, it shows that this virus circulates in this region and many times the infections occur in subclinical form in domestic species but can be fatal in wild population. Because the viral agents may travel large distances until to achieve wildlife habitats and could be a threat for those species and the biodiversity conservation, as that the lack of antibody in wild canids can cause increased mortality and reduced fertility when they are exposed this viral agent (De Almeida Curi et al., 2010; Cleaveland et al., 2000; Fiorello et al., 2004)

BAdV was found of expressive form in surface water. However, research into the presence its in environmental samples is limited yet, Even knowing that this virus is ubiquitous in bovines and your occurrence can cause since subclinical infections until abortion and cardiopathies (Spilki et al., 2009, Wong & Xagoraraki, 2010). A common practice in the rural environment is to use the manure bovine as fertilizer, but that is dangerous. Wong & Xagoraraki (2010) found significant levels of BAdV in manure, faces, and drainage water, that may indicate a hight potential of surface water pollution by the manure when applied to farm fields. The indicators of avian and porcine origins had a minor detection rate. This occurs can be because the production of these animals is not the main activity in this region, as the example, the pig farming that is mainly concentrated in the northwestern and eastern-central region of Rio Grande do Sul (IBGE, 2016).

For the viruses of human origin was realized detection of viral viability and considering the first and third passage in cells from groundwater and surface water, the rate of samples that had HAdV infectious was 13.7% (17/124). In surface water from Rio de Janeiro city of Brazil, was found infectious particles of HAdV in 17% of samples (Staggemeier et al., 2017). Those values are lower than already was seen in tap water that is destined for human consumption from Korea, where the rate of infectious particles of HAdV was 39.1% (Lee & Kim, 2002.).

However, even with a low rate of viability viral found, shouldn't discard the risk that the contact with that waters can present. Because the viruses evaluated are wild and they cannot adapt in cell culture and that way generates false negative results. Even if some studies show that the use of only qPCR essay are unable to estimate the number of variables particles in environmental samples (Leland & Ginocchio, 2007; Li, He & Jiang, 2010). Already was described that the difference is somewhat greater than 2 log when you compare the molecular detection before and after DNAse treatment, as was observed with the HAdV-2 (Girones et al., 2010). Furthermore, Bofill-Mas et al. (2006) performed a parallel essay with and without previous DNAse treatment and did not find notable differences too and that evidence suggesting that the particles were integrated. So, even if it were considered the minor concentration found ($9.40x10^4$) and the larger reduction (3 logs) considering the previous studies, those waters present risk.

Overall, the majority of AdV from animal origin had a higher detection rate in surface water. those water matrices suffering a sharp deteriorate in your quality through diffuse sources who are difficult to identify to adequate management and remediation planning. Among them, urban, forest and farming runoff, in which the last including the contamination by application of manure in the soil and the free access of cattle and other animals to rivers and streams (Do Amaral, 2004; Knappett et al., 2012; Staggemeier et al., 2015). In rural areas, those situations are acute, because the use of digesters has not been widely adopted yet, being common the use of landfills and direct disposal of wastewater on soil and water resources (Do Amara, 2004; De Oliveira et al., 2012; Spilki et al., 2013).

In rural regions, the groundwaters are the main sources of waters use to drink and others domestic needs (as, cleaner, showering, and kitchen). While preventive measures are rarely taken, as a representative evaluation of water quality from those underground sources (Gibson et al., 2011). There is yet a conviction that the groundwaters from the rural environments are safer and free of pathogenic microorganism, this way, have been often considered potable for human consumption (Do Amaral, 2004). This situation is potentially dangerous because the detection rates of HAdV and AvAdV were bigger than the rates found in surface water and the number of genomic copies was very similar in both matrices, being the maximum difference one log. Opposite what one imagines the aquifer contamination is common and can occur due to various factors (Howard et al., 2003; Gibson, 2014). The lack of basic

sanitation is one of the major contributors for this type of pollution because the environment contaminated allow leaching of some contaminants through the soil. Especially the enteric pathogens, who are able to migrate to deepest soil layers, reaching the groundwaters reserves by adsorption and desorption process (Knappett et al., 2012; Gotkowska-Płachta et al., 2015; Staggemeier et al., 2015).

Dissemination of enteric pathogens in groundwater already has been shown in aquifers of marine environments too. In this type of environments with little earth's surface, it is believed that the contamination of groundwaters occurs by displaced of the virus from septic tanks, in which was inferred that the virus can cover around 392 meters in 27 hours term (Futch, Griffin & Lipp, 2010). However, it is clear that the safe setback distance between wastewater disposal until the aquifers is hardly achieved. as already demonstrated by Blaschke et al. (2016) that evaluated small biological wastewater treatment systems and drinking water wells against virus contamination in alluvial aquifers. They concluded that the horizontal setback distances required for achieving 12-log reduction of the total numbers of enteric viruses is 39–144m in sand aquifers, 66–289 m in gravel aquifers and 1–2.5 km in coarse gravel aquifers.

The HAdVs (46.7%) detection rate is a considerable value. However, only it was possible to get this number because was used for more than one technique, in addition, the use of different oligonucleotides pairs with the same target (HAdV), but, with the different annealing regions. by the way, by doing the samples water passages at cell, following by molecular detection, was possible to observe that the number of positive samples becomes more expressive (54%), because submitting negative samples at cell culture with the goal of to increase the number of viable particles through its replication. This made the nine samples of which had been negative before of passages in cells, becoming positive. this situation can be explained by two hypotheses: a) the virus has been able to replicate in vitro and surpassed the detection minimum limit of the qPCR technique; b) after the samples passages in cell reduced the number of inhibitors of reaction qPCR. Those results caused an uncertainty about which are the most appropriate test, given that the results values may double when compared the results obtained of only an essay whit the technics set used in this research. The molecular tests are tools very useful to the identification of fecal contamination sources, because for some viruses that is the only form for detection and quantification method (Girones et al., 2010). However, it is difficult to elect a single

protocol to evaluated viral indicators in environmental matrices. Nowadays there are several different studies with different methods for HAdV detection available, however when it comes to detection in water, little is known about the sensitivity and specificity (Tong & Lu, 2011). This way, Ss the universal indicator probably not exist, each application or purpose must be to evaluated individually (Skraber et al., 2004).

4.5 CONCLUSION

The results of this study corroborate the findings of other already developed where show that the use only bacterium indicators is not enough to attest the microbiology quality of water. However, even if the AdVs have been considered excellent candidates to be used in conjunction with the current indicators, missing a standard protocol still. This results show that it is essential to discover a procedure that is fast, reliable, and that offsets the disadvantages of using only one diagnostic tool. Although the detection rate of infectious samples has not been so expressive, it is dangerous to speak that this water does not present an infection risk to health, since it was found a high concentration of genomic copies and it is already expected that some viruses from the environment do not adapt at cell cultures, probably underestimating the actual risk of infectious particles. In addition, these results demonstrate that the rural areas deserve more attention from government organs because it is evident that the basic sanitation is neglected in those locals.

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4. CONSIDERAÇÕES FINAIS

Tal como observado em estudos prévios, mesmo que *E. coli* seja um bom indicador de contaminação fecal e, muitas vezes o único parâmetro empregado em diversos países para atestar a qualidade microbiológica da água. A ausência dela, não exclui a presença de vírus entéricos. Desta maneira, é perceptível que a associação de mais de um tipo de indicador de contaminação fecal, a título de exemplo: *E. coli* e AdVs, tornaria a avaliação mais sensível, evitando resultados falsos-negativos. Além disso, foi possível observar que o HAdV foi o indicador viral mais abundante tanto em águas superficiais como também em subterrâneas. O que aponta para uma forte ação antrópica, onde demostra o quão o esgoto doméstico quando eliminado sem tratamento prévio, pode impactar a qualidade hídrica. Achado esse, observado em altos níveis nas águas subterrâneas, as quais supostamente seriam mais protegidas que as superficiais.

O estudo ainda ressalta a importância de desvendar uma ferramenta que seja capaz de compensar as desvantagens de utilizar somente uma técnica para a detecção de HAdV em água. Tendo em vista que foi demostrado que com a utilização de apenas uma técnica, o número real de contaminação pode ser subestimado, já que ao utilizar um conjunto de técnicas aumentou a sensibilidade e consequentemente o número de amostras positivas. Ademais, além das técnicas moleculares, o teste de viabilidade viral, o qual utilizou a técnica de cultivo celular integrado com qPCR, se mostrou uma boa ferramenta auxiliar no diagnóstico de vírus em água. Primeiramente, porque ela fornece dados de infecciosidade e, além disso, ela é capaz de amplificar partículas virais que podem estar muito diluídas em água, possibilitando que amostras que não tinham sido detectadas por técnicas moleculares, superassem o limite de detecção, diminuindo os resultados falsos-negativos.

A falta de saneamento básico que existe nas áreas rurais é um importante percursor da grande contaminação fecal encontrada nas águas do ambiente estudado. Esse fato, traz preocupação, pois nesses ambientes as águas subterrâneas servem como principal fonte para beber e, raramente tem sua potabilidade atestada ou então são tratadas. Desta forma, o estudo almeja alertar o descaso que existe em áreas rurais com as questões de saneamento, buscando uma maior atenção dos

órgãos públicos para promover medidas de correção e prevenção dessa adversidade. Visto que, a poluição fecal em fontes hídricas apresenta um risco a saúde humana e animal e favorece a degradação ambiental.

Além da contribuição acadêmica, os resultados desse trabalho foram apresentados e discutidos junto à equipe técnica da Emater/RS. Com propósito de avaliar individualmente cada propriedade, almejando melhorias para reduzir ou/e eliminar as fontes de contaminação das águas. Os técnicos de cada município receberam os laudos referentes a qualidade microbiologica da água de cada propriedade e, retornaram as elas com propostas de melhoramentos.

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5. ANEXO

Confirmação de submissão ao periódico Water Research.

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