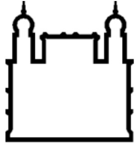

INSTITUTO OSWALDO CRUZ

Pós-Graduação em Biologia Celular e Molecular

TULIO MACHADO FUMIAN

DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE VÍRUS ENTÉRICOS EM ÁGUAS RESIDUÁRIAS NA CIDADE DO RIO DE JANEIRO

Tese apresentada ao curso de Pós-Graduação em Biologia Celular e Molecular do Instituto Oswaldo Cruz, FIOCRUZ, como parte dos requisitos para obtenção do título de Doutor em Ciências. Área de Concentração: Virologia

Orientadores: Dra. Marize Pereira Miagostovich
Dr. José Paulo Gagliardi Leite

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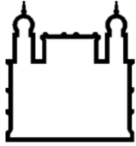

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Rio de Janeiro, 4 de Novembro de 2011

"Ao avaliarmos nosso progresso como indivíduos, tendemos a nos concentrar em fatores externos, como posição social, influência e popularidade, riqueza e nível de instrução. Certamente são dados importantes para se medir o sucesso nas questões materiais, e é perfeitamente compreensível que tantas pessoas se esforcem tanto para obter todos eles. Mas os fatores internos são ainda mais decisivos no julgamento do nosso desenvolvimento como seres humanos. Honestidade, sinceridade, simplicidade, humildade, generosidade pura, ausência de vaidade, disposição para ajudar os outros – qualidades facilmente alcançadas por todo indivíduo – são os fundamentos da vida espiritual."

Nelson Mandela, 1975.

"A vida é como andar de bicicleta. Para manter o equilíbrio, é preciso se manter em movimento"

Albert Einstein

À minha família.

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LISTA DE SIGLAS E ABREVIATURAS

aa – Aminoácido
CI – Controle interno
DNA – Ácido desoxirribonucléico
dsRNA – RNA de fita dupla
EIE – Ensaio imunoenzimático
EV - Enterovírus
ETE – Estação de tratamento de esgoto
Fiocruz – Fundação Oswaldo Cruz
G – Genogrupo
GA – Gastroenterite aguda
CG/L – Cópias de genoma por litro
HAdV – Adenovírus humanos
HAstV – Astrovírus humano
HAV – Vírus da hepatite A, do inglês “Hepatitis A Virus”
HEV – Vírus da hepatite E, do inglês “Hepatitis E Virus”
ICTV – Comitê Internacional de Taxonomia dos vírus, do inglês “International Committee on Taxonomy of Viruses”
kb – Kilobase
JCPyV – Poliomavírus JC
L – Litro
LVCA – Laboratório de Virologia Comparada e Ambiental
MS – Ministério da Saúde
ME – Microscopia eletrônica
mg/L – Miligrama por litro
nm – Nanômetros
nt – Nucleotídeo
NoV – Norovírus
NSP – Proteína não estrutural, do inglês “Non Structural Protein”
^{o}C – Grau Celsius
OMS – Organização Mundial da Saúde
ORF – Fase aberta de leitura, do inglês “Open Reading Frame”
P – Domínio externo da proteína capsídica VP1
PCR – Reação em cadeia pela polimerase, do inglês “Polymerase Chain Reaction”
pH – Potencial hidrogeniônico
PNI – Programa Nacional de Imunização
Poli(A) – Sequência repetitiva de nucleotídeos de adenina
qPCR – PCR quantitativa
RNA – Ácido ribonucléico
RpRd – RNA polimerase RNA dependente
RVA – Rotavírus espécie A
S – Domínio interno da proteína capsídica VP1
SaV - Sapovírus
T – Número de triangulação
UTR – Região não traduzida, do inglês “Untranslated Region”
UV – Ultravioleta
VLP – Partículas semelhantes a vírus, do inglês “Virus-like Particles”
VPg – Proteína viral do genoma, do inglês “Viral protein genome”

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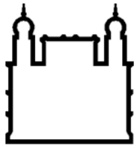

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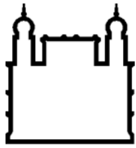
DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE VÍRUS ENTÉRICOS EM ÁGUAS RESIDUÁRIAS NO RIO DE JANEIRO

Tulio Machado Fumian

RESUMO

Entre a grande diversidade de vírus que infectam o homem, muitos são excretados em grandes concentrações nas fezes ou urina de indivíduos sintomáticos ou assintomáticos, estando presentes em águas residuárias provenientes de esgotos domésticos. Metodologias moleculares de amplificação genômica vêm sendo utilizadas para detecção e quantificação de vírus nestas águas, de modo que os estudos de virologia ambiental representam uma ferramenta a mais na determinação dos vírus circulantes em uma dada área geográfica. Este estudo teve como objetivo avaliar a disseminação dos principais vírus responsáveis pela gastrenterite aguda infantil [rotavírus A (RVA), norovírus (NoV) e astrovírus humanos (HAstV)], em águas residuárias da cidade do Rio de Janeiro, assim como avaliar os adenovírus humanos (HAdV) e poliomavírus JC (JCPyV) como marcadores virais de contaminação ambiental. Com este propósito, metodologias de concentração, detecção, quantificação e caracterização molecular de vírus foram estabelecidas e aplicadas em águas residuárias de duas Estações de Tratamento de Esgoto (ETE) da cidade. Inicialmente, realizou-se o monitoramento de RVA, NoV, HAstV e JCPyV na ETE Fiocruz (2004-2005) com a utilização do método de concentração viral baseado na ultrafiltração com membrana carregada negativamente. Nestes estudos demonstrou-se a disseminação destes vírus em amostras de esgoto bruto e em menor escala em esgoto tratado, assim como se avaliou as taxas de recuperação de NoV e HAstV pelo método de concentração utilizado e metodologias de detecção e quantificação viral. A caracterização dos agentes virais detectados demonstrou a circulação dos genótipos mais prevalentes (RVA G1 e P[8]; NoV GII; HAstV-1 e JCPyV tipo-3). As baixas taxas de recuperação viral obtidas com o método de filtração, assim como a ausência de controle interno nos estudos anteriores, apontaram a necessidade de se estabelecer diferentes métodos para investigações. Deste modo, estabeleceu-se o método de concentração de vírus por ultracentrifugação, assim como um protocolo de quantificação do bacteriófago PP7, utilizado como controle interno, e outro para quantificação de RVA. Estes métodos foram aplicados em um novo monitoramento (2009-2010), realizado em uma ETE de grande porte (ETE Alegria) visando obter dados mais consistentes dos vírus circulantes na população do Rio de Janeiro. Os resultados provenientes deste monitoramento demonstraram que os RVA, HAdV e os JCPyV foram os mais prevalentes nas amostras de águas residuárias seguidos pelos NoV e HAstV. A caracterização molecular demonstrou a diversidade de genótipos de vírus circulantes em concordância com os obtidos anteriormente, exceto para os RVA. RVA G2 e P[4] foram caracterizados em 100% das amostras de esgoto bruto revelando uma alteração do perfil dos genótipos circulantes após a introdução da Rotarix® no país em 2006. Neste estudo não foram detectadas cepas vacinais de RVA. Os resultados obtidos na ETE Alegria demonstraram uma redução de 2 logs na carga viral após o tratamento. Os dados quantitativos confirmaram o potencial do HAdV como marcador viral de contaminação, assim como revelaram a importância de se avaliar a descarga local de vírus no ambiente, de modo a se identificar os vírus presentes em altas concentrações que devem ser considerados em estudos de avaliação de risco.



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MOLECULAR DETECTION AND CHARACTERIZATION OF ENTERIC VIRUSES FROM WASTEWATERS IN RIO DE JANEIRO

Tulio Machado Fumian

ABSTRACT

Among the wide variety of viruses that infect humans, many are excreted in high concentrations in feces or urine of symptomatic or asymptomatic, being present in wastewater from domestic sewage. Molecular genomic amplification methods have been used for detection and quantification of virus in these waters, so studies of environmental virology represent another tool in the determination of circulating viruses in a given geographic area. This study aimed to evaluate the spread of the main viruses responsible for infantile acute gastroenteritis [rotaviruses A (RVA), norovirus (NoV) and human astrovirus (HAstV)], in wastewater from the city of Rio de Janeiro, as well as evaluating the human adenovirus (HAdV) and polyomavirus JC (JCPyV) as markers of viral contamination. For this purpose, methods of concentration, detection, quantification and molecular characterization of viruses were established and applied in two wastewater treatment plants (WTP) in this city. Initially, the monitoring of RVA, NoV, HAstV and JCPyV at WTP located in Fiocruz (2004-2005) was performed using viral concentration method based on ultrafiltration with negative charged membrane. In these studies, it was demonstrated the spread of these viruses in raw sewage samples and, to a lesser extent, in treated sewage, as well as evaluated the recovery rates of NoV and HAstV by the concentration method used and methods of viral detection and quantification. Characterization of viral agents detected demonstrated the circulation of the most prevalent genotypes (RVA G1 and P[8]; NoV GII; HAstV-1 and JCPyV type-3). The low recovery rates obtained with the viral filtration method, as well as the lack of internal control in previous studies, indicate a need to establish different methods for future investigations. Thus, we established the method of virus concentration by ultracentrifugation, as well as a protocol for the quantification of bacteriophage PP7, used as an internal control, and another for RVA quantification. These methods were employed in a new monitoring (2009-2010), held in a large WTP (WPT Alegria) to obtain consistent data from viruses circulating in the population of Rio de Janeiro. The results from this monitoring showed that RVA, HAdV and JCPyV were the most prevalent in wastewater samples, followed by NoV and HAstV. The molecular characterization demonstrated the diversity of genotypes circulating in agreement with those obtained previously, except for the RVA. RVA G2 and P[4] were characterized in 100% of raw sewage samples, and revealed a change in the profile of circulating genotypes after the introduction of Rotarix® in the country of 2006. In this study RVA vaccine strains were not detected. The results in WTP Alegria demonstrated a viral load reduction of about two logs. The quantitative data confirmed the potential of HAdV as a viral marker of contamination, and revealed the importance of evaluating the discharge of virus in the local environment in order to identify viruses present in high concentrations to be considered in assessment studies risk.

1. INTRODUÇÃO

O impacto global das doenças de veiculação hídrica e das doenças relacionadas à água é extremamente difícil de ser avaliado, principalmente pela falta de dados e estudos epidemiológicos e da presença de inúmeras variáveis. Outras razões que podem ser destacadas incluem a dificuldade de se confirmar a fonte de infecção, infecções sub-clínicas e/ou assintomáticas e a transmissão secundária destas (Gerba et al. 1996). Consequentemente, estimativas do real impacto das doenças de veiculação hídrica na saúde da população variam principalmente de acordo com a região/país analisada.

Atualmente, os vírus são os principais agentes causadores de doença de veiculação hídrica e doenças relacionadas à água (Grabow 2007). A cada dia, as doenças diarréicas de fácil prevenção matam aproximadamente 5.000 crianças no mundo, fato que poderia ser amenizado com o maior acesso à água potável e redes de saneamento (WHO 2005).

A busca por patógenos virais em ecossistemas aquáticos iniciou-se após a ocorrência de um surto de hepatite em Nova Délhi (Índia), na década de 50, como conseqüência da contaminação do sistema de tratamento da água por patógenos virais provenientes de águas residuárias. A epidemia que atingiu cerca de 230.000 pessoas foi causada pelo vírus da hepatite E (HEV), sendo este agente identificado anos após o episódio (Bosch 1998). Outros surtos envolvendo um grande número de indivíduos foram relatados posteriormente. Em 1988, em Xangai, o consumo de frutos de mar cultivados em água estuarina contaminada com esgoto resultou em um surto de 300.000 casos de hepatite, causada pelo vírus da hepatite A (HAV) e 25.000 casos de gastroenterite (Halliday et al. 1991). Em 1991, um surto de aproximadamente 79.000 casos de hepatite causado pelo HEV foi descrito em Kampur, India, pelo consumo de água potável contaminada (Ray et al. 1991).

A investigação da qualidade microbiológica de água tem sido baseada no estudo de microorganismos indicadores tais como coliformes fecais, *Escherichia coli* e *Enterococcus*. Contudo a utilização desses indicadores para predizer a presença de vírus tem sido questionada, demonstrando a falta de correlação existente entre essas duas classes de microorganismos. Diversos agentes virais têm sido freqüentemente detectados em águas que se encontram dentro dos padrões bacteriológicos (Lipp et al. 2001; Tree et al. 2003; Vivier et al. 2004; Sinclair et al. 2009). Além do fato de indicadores bacterianos serem mais sensíveis à inativação pelos processos de tratamento e pela luz solar quando comparados aos patógenos virais e protozoários, outras limitações da utilização de padrões bacterianos podem ser discutidas, tais como: a excreção fecal não é exclusiva de humanos; habilidade de multiplicar em alguns ambientes; menor tempo de permanência em ambientes aquáticos; a incapacidade

de identificação da fonte de contaminação e a baixa correlação com a presença de outros patógenos (Pina et al. 1998; Scott et al. 2002; Simpson et al. 2002; Field et al. 2003; Horman et al. 2004; Pote et al. 2009; Girones et al. 2010).

Neste contexto, a pesquisa de vírus em diferentes matrizes aquáticas, tais como águas de consumo, superficiais (fluviais e marinhas) ou residuárias (Miagostovich et al. 2008; Katayama et al. 2008; Albinana-Gimenez et al. 2009; Haramoto et al. 2010) e a tentativa de se estabelecer um marcador viral de contaminação humana (Pina et al. 1998) tem aumentado o número de publicações na área de virologia ambiental.

1.1 Águas residuárias

Águas residuárias são constituídas por uma mistura de excretas de origem humana e de animais, sólidos suspensos e uma variedade de substâncias químicas originárias de atividades residenciais, comerciais e industriais (Argaw 2004).

A remoção viral de águas residuárias continua recebendo atenção devido à importância epidemiológica dos vírus presentes como patógenos de transmissão hídrica e devido à grande diversidade de agentes excretados nos dejetos humanos (Rose et al. 1996). Altas concentrações de vírus entéricos são excretadas nas fezes e urina de indivíduos, o que faz do esgoto bruto (sem tratamento) ser considerado o maior carreador de agentes causadores de doenças, particularmente de patógenos entéricos virais. Durante o pico de infecção, tem sido descrito que indivíduos podem excretar vírus entéricos em níveis elevados (até 10^{11} vírus/grama de fezes), e de acordo com trabalhos realizados em diferentes regiões do mundo, a concentração viral em amostras de esgoto bruto e tratado alcança aproximadamente 10^4 – 10^9 e 10^2 – 10^7 vírus por litro, respectivamente (Laverick et al. 2004; Bofill-Mas et al. 2006; Carducci et al. 2008; Fong et al. 2010; Fumian et al. 2010; Girones et al. 2010; Kuo et al. 2010; Rodriguez-Manzano et al. 2010; Simmons & Xagoraraki 2011).

Existem várias rotas de exposição e contaminação por vírus entéricos podendo estes ser adquiridos, principalmente após o consumo de água ou de alimentos contaminados (Figura 1). A descarga de agentes virais via águas residuárias *in natura* ou pelo descarte de efluentes de estações de tratamento de esgoto (ETEs) em ambientes aquáticos coloca em risco de infecção a população exposta, por contato com águas recreacionais contaminadas ou acúmulo de agentes patogênicos em frutos do mar, como ostras e outros tipos de moluscos bivalves (Metcalf et al. 1995; Carducci et al. 2008).

Os tratamentos aplicados às águas residuárias antes de seu despejo no meio ambiente visa remover contaminantes, tanto químicos quanto biológicos, diminuindo os impactos

negativos no ecossistema receptor (Okoh et al. 2010). O papel exercido pelas ETEs deve ser enfatizado como um dos meios mais efetivos de reduzir a concentração de vírus excretados para o meio ambiente contribuindo diretamente para redução do risco de infecções via água contaminada (Carducci et al. 2008; Haramoto et al. 2008; Katayama et al. 2008).

Entretanto, como conseqüência da dificuldade da total eliminação destes patógenos pelos sistemas de tratamento de esgoto, estes podem se dispersar para ecossistemas aquáticos em concentrações suficientes para colocar em risco a saúde humana (Bosch et al. 2008; Cantalupo et al. 2011).

Os processos físicos como sedimentação, lodo ativado e filtros, aplicados nas ETEs municipais são capazes de remover em torno de 90-99% (equivalente a 1 – 2 logs) da carga viral presente nas águas residuárias (Ueda & Horan 2004; Okoh et al. 2010). A remoção adicional de contaminantes microbiológicos (idealmente 99,99% ou 4 logs de redução) é dependente de posterior desinfecção em tratamentos terciários com cloro, UV e ozônio (Templeton et al. 2004; Mezzanotte et al. 2007). Estudos anteriores demonstraram a maior eficiência de inativação viral para o processo de tratamento com cloração em relação à utilização da luz UV (Simonet & Gantzer 2006a,b; Rodriguez et al. 2008; Simmons et al. 2011). Apesar da combinação de todos os processos utilizados em ETEs, a eficiência de remoção da carga viral varia entre os processos aplicados, resultando na descarga destes agentes nos efluentes das plantas para o meio ambiente, como demonstrados por diversos estudos (Formiga-Cruz et al. 2002; Griffin et al. 2003; Arraj et al. 2005; Fong & Lipp 2005; Carducci et al. 2008).

Atualmente, o monitoramento de águas residuárias também é considerado uma ferramenta importante para o entendimento da incidência dos agentes virais em determinadas áreas geográficas. A identificação de vírus em esgoto bruto representa uma importante fonte de dados para estudos de epidemiologia e distribuição viral, gerando informações concretas a respeito dos genótipos circulantes na comunidade, maior ou menor circulação de determinado agente viral num período de tempo, sazonalidade e distribuição geográfica (Laverick et al. 2004; Bosch et al. 2008; Haramoto et al. 2008; Lee and Kim 2008; Sinclair et al. 2008).

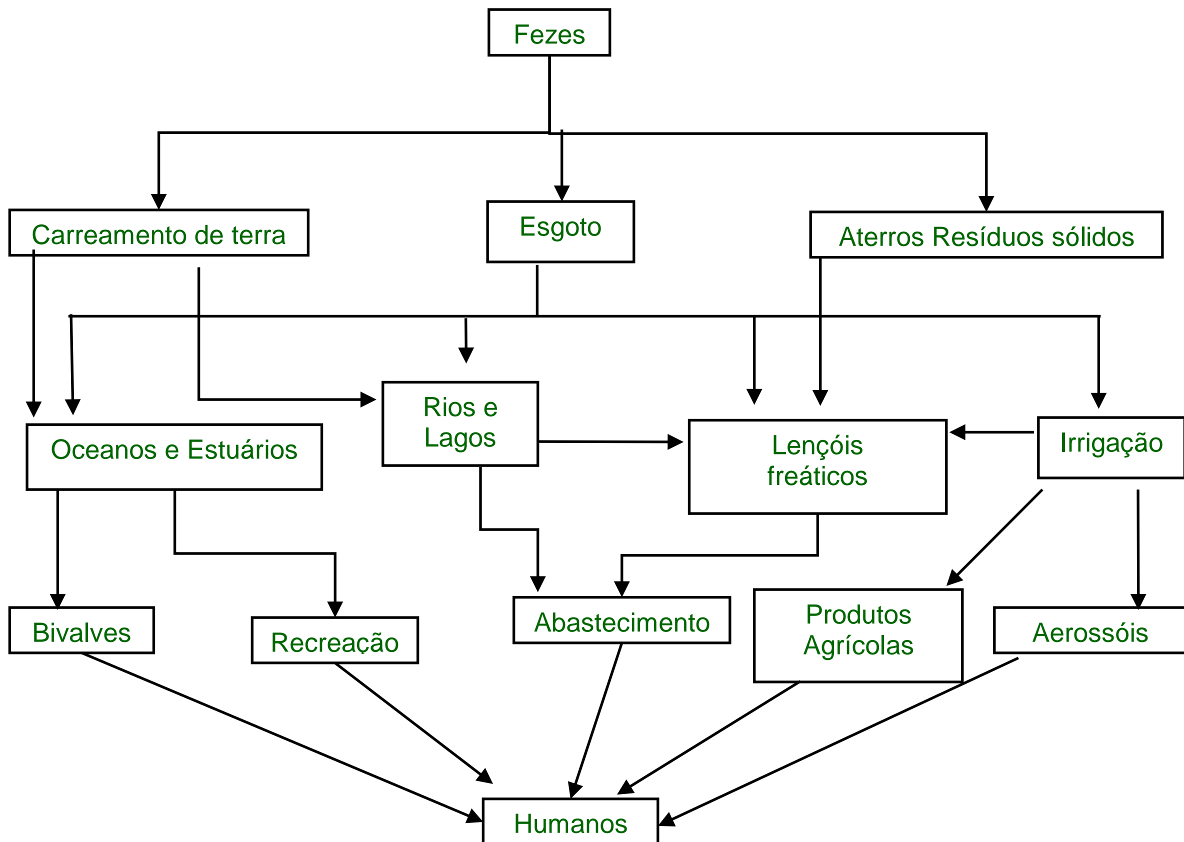

Figura 1: Rotas de transmissão de vírus entéricos (Modificado de Metcalf et al. 1995).

1.2 Metodologias de concentração e detecção viral

O procedimento para análise de vírus em amostras de águas ambientais envolve diferentes etapas, tais como: coleta, clarificação, concentração, descontaminação/remoção de inibidores, seguido de um processo de detecção apropriado. O volume a ser analisado varia e depende, basicamente, da origem da amostra. Enquanto que para amostras de água residuárias é possível detectar vírus em um volume igual ou inferior a 100 mL, para amostras de águas recreacionais o volume pode chegar a 10 L, necessitando de uma concentração de 1000 vezes para se obter um volume reduzido para detecção viral (Wyn-Jones 2007).

Diferentes metodologias de concentração baseadas nas propriedades dos vírus vêm sendo utilizadas tais como: adsorção/eluição (carga iônica da partícula viral), a ultrafiltração (tamanho da partícula) e a ultracentrifugação (densidade e coeficiente de sedimentação) (Pina et al. 1998; Wyn-Jones & Sellwood 2001; Katayama et al. 2002). Um bom método de concentração deve ser tecnicamente simples, rápido, de baixo custo, além de promover a recuperação de uma grande variedade de vírus, gerar um pequeno volume de concentrado e

ser reprodutível (Bosch 1998; Wyn-Jones & Sellwood 2001). Contudo, nenhum método atende a todos esses critérios (Wyn-Jones 2007).

A associação de metodologias de concentração viral com técnicas moleculares de detecção representou, nos anos 80, um avanço nos estudos de virologia ambiental. Embora não permita diferenciar entre partículas infecciosas e não infecciosas, a detecção do genoma viral é considerada por muitos investigadores uma boa técnica para monitoramento principalmente por permitir a detecção de vírus fastidiosos ou daqueles que não se propagam em cultura celular (Girones et al. 2010).

A técnica molecular mais utilizada atualmente é a reação em cadeia pela polimerase (PCR), que quando comparada ao isolamento viral em cultura de células, apresenta diversas vantagens tais como: a) redução no tempo e facilidade de execução; b) sensibilidade; e c) baixo custo (Abbaszadegan et al. 1999; Schvoerer 2000; Gofti-Laroche et al. 2001; Carducci et al. 2003). Além disso, a baixa estabilidade do ácido ribonucléico (RNA) livre no ambiente sugere que este método detecte partículas virais intactas e não o genoma viral livre da partícula (Abbaszadegan et al. 1999; Wyn-Jones & Sellwood 2001; Gofti-Laroche et al. 2001; Carducci et al. 2003). Uma variante da PCR, a técnica quantitativa (qPCR), apresenta todas as características citadas anteriormente, com a vantagem de gerar resultados quantitativos que possibilitam a realização de estudos de avaliação de risco microbiológico, além de permitir avaliar a eficiência dos métodos de tratamento por redução logarítmica do número de genomas detectados antes e após o tratamento (Smeetis et al. 2010).

A concentração dos vírus é uma etapa crítica, pois resulta na concentração de diferentes substâncias presentes na água, sendo relevante uma adequada associação do método de concentração viral com as metodologias de detecção utilizadas. Inúmeras substâncias, tais como ácido húmico e fúlvico presentes na água têm a capacidade de inibir a atividade das enzimas utilizadas nas metodologias moleculares de detecção, gerando resultados falsos negativos (Ijzerman et al. 1997; Katayama et al. 2002). A utilização de um controle interno (CI), baseado na inoculação de bacteriófago anteriormente à etapa de concentração viral, vem sendo considerado uma importante ferramenta para excluir reações falso-negativas (Hoorfar et al. 2004; Rajal et al. 2007).

1.3 Vírus de veiculação hídrica

Existem mais de 140 diferentes tipos de vírus encontrados nos dejetos humanos e animais e todos são potencialmente transmitidos pela água (Gerba et al. 1996; Sinclair et al. 2009). Os vírus entéricos são mais resistentes à inativação do que os indicadores bacterianos

fecais, sendo encontrados em águas onde os níveis bacterianos estavam dentro dos padrões de qualidade (Griffin et al. 1999). A alta concentração e os diferentes tipos de vírus detectados em águas residuárias ou em ambientes contaminados por lançamentos de esgoto sem tratamento demonstram o fluxo de vírus na população e refletem as infecções virais mais prevalentes na comunidade, além do nível de poluição da água (Laverick et al. 2004; Girones et al. 2010).

Diversas famílias virais têm sido descritas presentes em ecossistemas aquáticos, tais como: *Adenoviridae* [adenovírus humanos (HAdV)], *Picornaviridae* [enterovírus (EV) e HAV], *Reoviridae* [rotavírus A (RVA)], *Caliviridae* [norovírus (NoV) e sapovírus (SaV)], *Hepeviridae* (HEV) e *Poliomaviridae* [poliomavírus JC (JCPyV)]. Estes agentes virais humanos são causadores de diversas infecções como gastroenterite, conjuntivite, infecções do trato respiratório, hepatite, e outras sérias infecções como meningite, miocardite, encefalite e paralisia (Sinclair et al. 2009).

Atualmente, o único parâmetro viral incluído nas normas ambientais é a presença de EV em águas recreacionais que, em geral, se isolam e quantificam com facilidade em linhagens celulares (Lucena et al. 1986). Os dados obtidos utilizando técnicas moleculares têm permitido observar que a presença de EV não se relaciona com a presença de outros agentes virais e se tem sugerido a utilização de HAdV e JCPyV como índice molecular de contaminação viral de origem humana (Grabow et al.1995; Pina et al.1998; Formiga-Cruz et al. 2003, Boffil-Mas et al. 2006). Deste modo, os HAdV e os JCPyV, juntamente com os principais agentes virais responsáveis pela gastroenterite aguda (GA) (RVA, NoV e HAstV) serão objetos deste estudo.

1.3.1 Adenovírus humanos

Os adenovírus humanos (HAdV) são classificados dentro da família *Adenoviridae,* gênero *Mastadenovirus*. São vírus não envelopados, com capsídeo de simetria icosaédrica, tamanho aproximado de 90 nm de diâmetro e contendo genoma de DNA dupla fita com aproximadamente 35 kb (Brown et al. 1996). A proteína do capsídeo é composta de 92 capsômeros – 240 *hexons* e 12 *pentons* – e estruturas chamadas fibras que se projetam de cada *penton* para o exterior (Berk 2007). Os *hexons* são formados por proteínas que participam da montagem e confere estabilidade à partícula viral (Figura 2). As proteínas do *penton* têm a função de penetração celular e as fibras são as responsáveis pela ligação das partículas virais ao receptor celular. O *core* viral é formado por pelo menos oito proteínas, tendo a função de manter a integridade do genoma e participar de atividades enzimáticas (Berk 2007).

Atualmente, 51 sorotipos diferentes de HAdV, agrupados em 6 espécies (A–F) são descritos baseado em ensaios com anticorpos neutralizantes e a habilidade de aglutinar hemácias (Berk 2007).

Os HAdV causam uma variedade de manifestações clinicas incluindo gastroenterite, doença respiratória aguda, pneumonia e conjuntivite epidêmica. Podem estabelecer infecções latentes e persistentes, sendo os vírus excretados por semanas após a infecção e independente do sítio primário da infecção, os HAdV são geralmente excretados pelas fezes em altas concentrações (Berk 2007; Mena & Gerba 2009). Tem sido descrito que a transmissão fecal-oral é responsável pela grande parte das infecções por HAdV em crianças pequenas, independente do sítio primário da infecção. Em adição, além dos HAdV entéricos 40 e 41, outros sorotipos que são considerados "não entéricos" por se replicarem no intestino, são potencialmente capazes de serem transmitidos por água contaminada (Flomenberg 2005; Mena & Gerba 2009).

As infecções causadas por HAdV ocorrem no mundo todo e durante todos os meses do ano (Flomenberg 2005). Diversos estudos têm demonstrado que os HAdV apresentam alta estabilidade sob condições de estresse ambiental, como radiação UV, temperatura, concentração de cloro e variações de pH e são comumente encontrados em águas ambientais (Pina et al. 1998; Gerba et al. 2002; Thompson et al. 2003; Carter 2005; Sedmak et al. 2005). Essas características têm colocado os HAdV como um dos principais candidatos à marcador viral de contaminação fecal de origem humana (Pina et al. 1998; Hundesa et al. 2006).

Os HAdV são, atualmente, os vírus DNA mais bem caracterizados (Haramoto et al. 2010). Diversos estudos realizados em diferentes regiões do mundo têm utilizado técnicas moleculares para detecção do genoma dos HAdV em amostras ambientais incluindo: água de piscina, águas superficiais, águas residuárias, águas de rio e moluscos bivalves (Papapetropoulou &Vantakaris 1998; Pina et al. 1998; Chapron et al. 2000; Jiang et al. 2001; Bofill-Mas et al. 2006; Calgua et al. 2008; Katayama et al. 2008; Albinana-Gimenez et al. 2009; Hamza et al. 2009; Fong et al. 2010; Haramoto et al. 2010; Schlindwein et al. 2010).

Surtos de veiculação hídrica relacionados às infecções por HAdV têm sido relatados desde a década de 50, principalmente em águas recreacionais de piscinas, sendo identificados 13 surtos publicados, causados por HAdV, nas ultimas 6 décadas de estudo (Sinclair et al. 2009).

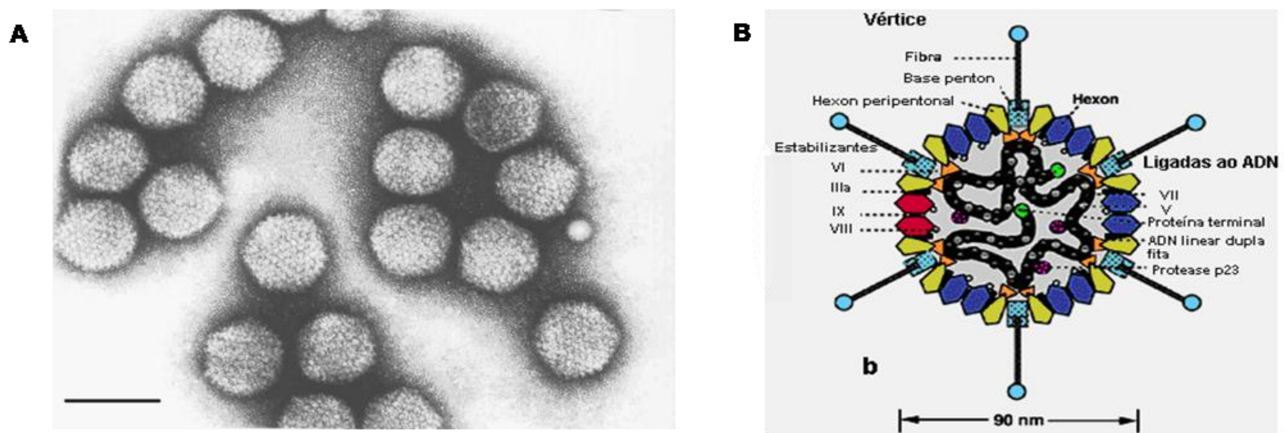

Figura 2: Partícula de adenovírus. A) Microscopia eletrônica de transmissão. B) Esquema representativo dos componentes e DNA. Os constituintes do virion são designados pelos números de seus polipeptídios com exceção da proteína terminal (TP). A partícula de adenovírus é composta de um capsídeo externo e de um DNA dupla fita linear associado ao core, duas proteínas terminais, as proteínas V e VII condensadas ao DNA e aproximadamente dez cópias da protease cistina p23 ligadas a ele. É ligada à parede interna do capsídeo pela proteína VI. O capsídeo é constituído principalmente por proteínas do hexon e é estabilizado através das proteínas IIIa, VIII e IX. Os vértices do capsídeo são compostos pelo penton, base do penton e fibras (A - http://www.ictvdb.rothamsted.ac.uk/Images/Cornelia/adeno_em.htm; B - Adaptado de Berk 2007). Barra = 100nm.

1.3.2 Poliomavírus JC

Inicialmente os poliomavírus foram classificados como um gênero dentro da família *Papovaviridae*, juntamente com os papilomavírus. Contudo, no ano 2000, o Comitê Internacional de Taxonomia de Vírus (ICTV) separou formalmente os dois grupos em duas famílias: *Poliomaviridae* e *Papilomaviridae*. Os JCPyV pertencem ao gênero *Poliomavirus*, e juntamente com os poliomavírus BK (BKPyV) constituem os poliomavírus que infectam humanos.

São vírus não envelopados, medindo cerca de 40-45 nm de diâmetro, contendo genoma composto de DNA circular dupla fita de aproximadamente 5,1 kb dividido em três regiões: região inicial, que codifica para proteínas expressadas com funções anteriores ao início da replicação do DNA; região tardia, codificando para proteínas com funções exercidas após o início da replicação e a região regulatória, que contém a única origem de replicação de DNA, assim como o promotor para a transcrição dos genes inicial e tardio. O genoma dos poliomavírus codifica para duas proteínas não estruturais principais (antígeno T maior e antígeno T menor) e três proteínas estruturais que compõe o capsídeo viral: VP1, 2, 3. O

capsídeo icosaédrico é composto por 72 pentâmeros, cada um contendo cinco moléculas de VP1 e uma de VP2 e VP3 (Imperiale & Major 2007).

A transmissão dos poliomavírus humanos ainda permanece desconhecida. Um estudo descreveu a capacidade dos JCPyV se replicarem em linfócitos B localizados nas tonsilas e células do estroma, reforçando a hipótese de que o trato respiratório seria o sítio primário de infecção e replicação viral (Monaco et al. 1998).

Os JCPyV são detectados em altas concentrações em amostras de águas residuárias ou em amostras ambientais contaminadas com esgoto em diferentes regiões do mundo (Bofill-Mas et al. 2000; 2006; Fumian et al. 2010; Haramoto et al. 2010). Esse alto padrão de excreção por via urinária e a frequente detecção dos poliomavírus em amostras ambientais indicam a possibilidade de transmissão viral por via urino-oral ou por ingestão de água contaminada (Bofill-Mas et al. 2000). Autores defendem a hipótese de transmissão intra familiar ou pessoas com relacionamentos estreitos e durante os primeiros anos de vida (Bofill-Mas et al. 2000; Zheng et al. 2004).

A infecção primária geralmente ocorre durante a infância e é subclínica. Após a infecção, os vírus persistem assintomaticamente nos tecidos renais (Arthur & Shan 1989; Kitamura et al. 1990). A reativação dos JCPyV pode ocorrer em qualquer etapa da vida, como demonstrado pela detecção de vírus sendo excretado na urina de 20 – 80% de indivíduos adultos saudáveis (Agostini et al. 2001; Pavesi et al. 2005).

A distribuição dos genótipos de JCPyV na população mundial parece obedecer um padrão definido. Um genótipo específico excretado por indivíduos é determinado, em maior proporção por sua etnia e não pela região geográfica que esse indivíduo se encontra (Agostini et al. 1997; Bofill-Mas et al. 2000).

Diversas características, tais como: a alta prevalência de infecções na população mundial, o alto nível de detecção em diversas matrizes aquáticas, a grande estabilidade no meio ambiente e infecção exclusiva em hospedeiro humano fazem dos JCPyV, juntamente com os HAdV, bons candidatos a marcadores virais de contaminação humana (Puig et al. 1994; Pina et al. 1998; Bofill-Mas et al. 2000; 2006; Calgua et al. 2008; Albinana-Gimenez et al. 2009).

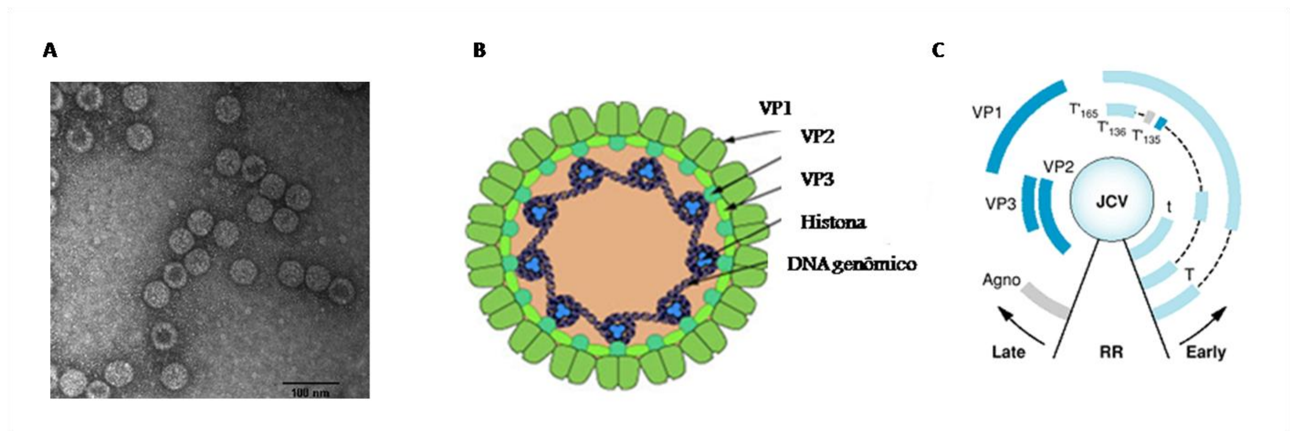

Figura 3: Partícula de *Poliomavírus*. A) Microscopia eletrônica de partículas de BKPyV. B) Reconstrução esquemática tridimensional. B) Esquema representativo de uma partícula de JCPyV baseada no conhecimento de seus polipeptídios e componentes de DNA. (Imperiale & Major 2007; http://viralzone.expasy.org/all_by_species/148.html). Barra = 100nm.

1.3.3 Rotavírus

Os rotavírus pertencem à família *Reoviridae*, gênero *Rotavirus*. A partícula viral não envelopada possui simetria icosaédrica, mede aproximadamente 70 nm de diâmetro e contém um RNA de fita dupla (dsRNA) com onze segmentos que codifica distintas proteínas (Estes & Kapikian 2007). Este perfil segmentado do genoma é facilmente identificado pela eletroforese em gel de poliacrilamida (EGPA), pela qual é possível visualizar diretamente os onze segmentos de RNA (Figura 4). Os segmentos genômicos variam em tamanho de 667 a 3.302 pares de bases (pb) e o genoma total contém aproximadamente 18.522 pb. O vírion possui três camadas protéicas: capsídeo externo, capsídeo interno e core, onde se situa o dsRNA. Associado ao core, encontram-se três proteínas estruturais: VP1, VP2 e VP3, codificadas pelos segmentos genômicos 1, 2 e 3, respectivamente. Estas moléculas representam, em conjunto, aproximadamente 18% das proteínas de uma partícula de rotavírus (Estes & Kapikian 2007).

O capsídeo interno é formado pela proteína VP6, codificada pelo segmento genômico 6. Esta é a proteína mais abundante e corresponde a 51% da porção protéica da partícula viral (Estes & Kapikian 2007). No capsídeo externo encontram-se as proteínas estruturais VP4, codificada pelo gene 4 e VP7, codificada pelos genes 7, 8 ou 9 dependendo da cepa (Estes & Kapikian 2007).

São reconhecidos sete grupos (espécies) de rotavírus, identificados pelas letras maiúsculas A, B, C, D, E, F e G (Bridger et al. 1994). Os rotavírus dos grupos A, B e C têm

sido encontrados tanto em humanos quanto em animais, enquanto que os grupos D-G foram identificados somente em animais.

Os rotavírus da espécie A (RVA) são os mais importantes, por serem os principais responsáveis pelos episódios de diarréia aguda em crianças em todo o mundo (Estes & Kapikian 2007). Diferentes sorotipos/genótipos foram identificados dentro da espécie A. Os antígenos que definem os sorotipos e genótipos estão presentes nas proteínas estruturais VP4 e VP7 do capsídeo externo e induzem a produção de anticorpos neutralizantes. Desta maneira, a denominação de sorotipo refere-se às duas especificidades, em relação à VP4 e/ou à VP7. Como VP7 é uma glicoproteína, a especificidade por ela conferida é denominada sorotipo G e, de maneira análoga, o fato de VP4 ser uma proteína sensível a uma protease faz com que a especificidade por ela determinada seja denominada sorotipo P. Sorotipos ou genotipos G são designados por algarismos arábicos.

A denominação de sorotipo/genótipo obedece, portanto, a um sistema binário do tipo GxP[y], podendo haver diversas combinações entre as especificidades G e P. Os RVA pertencentes aos genotipos G1P[8], G2P[4], G3P[8], G4P[8] e G9P[8] têm sido os mais identificados, numa escala global, como agentes etiológicos de diarréia em crianças menores que cinco anos de idade, causando cerca de 600.000 mortes por ano em crianças nesta faixa etária nos países em desenvolvimento (Parashar et al. 2003; 2006).

Os rotavírus são excretados em altas concentrações (até 10^{11} partículas/g) nas fezes de indivíduos infectados e podem persistir no ambiente por prolongados períodos de tempo (Carter 2005). Essa resistência às condições extremas do meio ambiente resulta no potencial de contaminação para águas recreacionais e fontes de água potável (Sinclair et al. 2009). Diversos surtos de GA causados por RVA devido ao contato com água contaminada têm sido relatados resultando em doença em crianças e adultos (Hung et al. 1984; Gerba et al. 1996; Kukkula et al. 1997; Villena et al. 2003).

Em março 2006, por recomendação da Organização Mundial da Saúde (OMS), o Ministério da Saúde (MS) incluiu a vacina atenuada anti-rotavírus G1P[8] (Rotarix®, GlaxoSmithKline, Rixensart, Bélgica) no calendário oficial de vacinações do MS, pelo Programa Nacional de Imunizações (PNI) (Carvalho-Costa et al. 2009). Estudos anteriores, em várias partes do mundo, demonstram flutuações temporais e geográficas na distribuição das combinações G-P de RVA (Santos & Hoshino 2005). Se a infecção primária contra RVA confere proteção principalmente genótipo específico, a eficácia protetora pela vacina poderá variar dependendo dos genótipos circulantes entre as diferentes populações e em uma determinada estação (Santosham et al. 1997). Estudos de Fase III com a vacina Rotarix® sugerem que, apesar de ser uma vacina monovalente, a mesma confere imunidade cruzada

contra outros genótipos (Ruiz-Palácios et al. 2006; Vesikari et al. 2006). Entretanto, deve-se ter o monitoramento epidemiológico de RVA circulantes anterior e posteriormente à introdução da vacina Rotarix® no Brasil, para ser avaliada a real eficácia desta vacina (Fase IV). Neste contexto, torna-se fundamental a análise dos genes estruturais (VP4, VP6 e VP7) dos diferentes genótipos de RVA detectados antes e após a introdução da vacina Rotarix®.

Estudos de virologia ambiental podem fortalecer o conhecimento e a vigilância dos genótipos circulantes de RVA na população, utilizando amostras de esgoto, e com isso, complementar os dados obtidos de estudos epidemiológicos utilizando amostras clínicas.

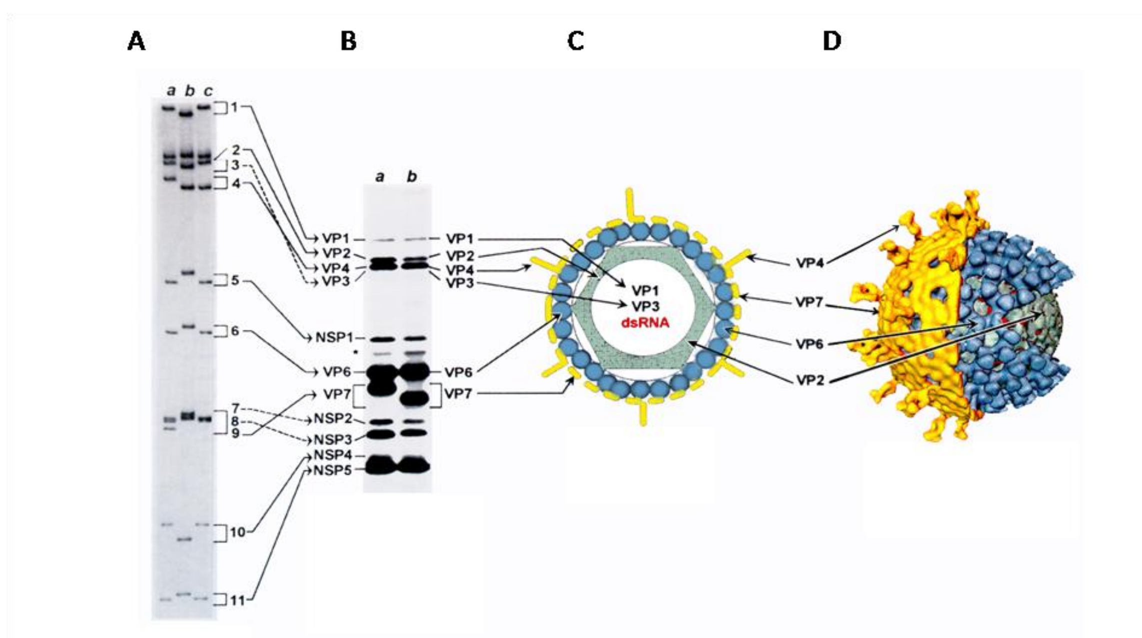

Figura 4: Partícula de rotavírus. A e B) Gel de acrilamida demonstrando os segmentos e as proteínas virais. C) esquema do virion. D) Reconstrução esquemática da superfície viral demonstrando as proteínas de superfície. (Modificado de Estes et al. 2001).

1.3.4 Norovírus

Os norovírus (NoV) pertencem ao gênero *Norovirus* da família *Caliciviridae*. São vírus não envelopados, constituídos por um RNA de fita simples e polaridade positiva de aproximadamente 7.700 nucleotídeos (nt). O genoma é organizado em três fases abertas de leitura (ORFs), região não traduzida (UTR) nas extremidades 3´ e 5´ e cauda poli(A) na extremidade 3'. Observa-se também um RNA subgenômico de aproximadamente 2.300 nt que

contém as ORFs 2 e 3, uma UTR e cauda poli(A) na extremidade 3´. A ORF1 codifica uma poliproteína, que após a clivagem proteolítica dá origem às proteínas não estruturais, incluindo a RNA polimerase RNA dependente (RpRd). As ORFs 2 e 3, localizadas na extremidade 3' do genoma codificam para as proteínas estruturais do capsídeo denominadas de VP1 (proteína principal) e VP2, respectivamente. Na extremidade 5´ do RNA genômico e subgenômico se encontra a proteína VPg unida covalentemente (Hardy 2005) (Figura 5).

O capsídeo viral icosaédrico é formado por 90 dímeros da proteína VP1 que se divide em dois domínios principais designados S e P. O domínio S contém elementos essenciais para a formação da parte interna do capsídeo icosaédrico enquanto o domínio P interage em contatos diméricos com o domínio S aumentando a estabilidade da partícula e formando as proeminências no capsídeo viral. Este domínio P está dividido em dois subdomínios denominados P1 e P2. O subdomínio P2 forma a parte externa do capsídeo viral que está inserida no subdomínio P1. A região hipervariável no subdomínio P2 tem um papel importante no reconhecimento do receptor celular e na imunogenicidade do vírion (Hardy 2005).

O gênero *Norovirus* apresenta grande diversidade genética e antigênica. Baseado na sequência de informação gênica da VP1, os NoV podem ser classificados em cinco diferentes genogrupos (GI, GII, GIII, GIV e GV), dos quais GI, GII e GIV são detectados em humanos (Zheng et al. 2006). A grande diversidade dos NoV é atribuída tanto ao acúmulo de erros gerados pela RpRd durante a replicação, quanto da ocorrência de recombinação viral durante uma co-infecção (Bull et al. 2007; Zheng et al. 2010).

Estudos de caracterização molecular dos NoV associados a casos de GA revelam uma frequente co-circulação de diferentes genótipos de GI e GII, porém o genótipo 4 do genogrupo II (GII.4) é o mais prevalente, sendo responsável por 75% a 100% dos genótipos caracterizados em surtos de GA (Green 2007; Glass et al. 2009; Siebenga et al. 2009; Zheng et al. 2010).

Um importante fator a ser considerado nas infecções pelos NoV é a susceptibilidade genética da população. Estudos demonstram que determinantes genéticos de resistência do hospedeiro à infecção estão relacionados à presença de receptores virais na superfície celular presentes na mucosa intestinal (Hutson et al. 2004; Tan & Jiang 2005). Os antígenos do grupo histo-sanguíneo ABO têm sido relacionados à susceptibilidade a infecção por determinados genótipos de NoV. Como demonstrado previamente, indivíduos do grupo histo-sanguíneo B raramente apresentam evidências de infecção pelo genótipo GI.1 (Lindesmith et al. 2003; Green 2007).

Os surtos de GA causados por NoV ocorrem principalmente em ambientes fechados como casas de repouso, hospitais, asilos, creches, navios, hotéis e restaurantes. Apesar de

envolverem indivíduos de todas as idades, esses surtos são particularmente importantes em crianças pequenas e idosos, podendo ocorrer agravamento dos sintomas, e até mesmo o óbito, como demonstrado por estudos realizados em diferentes países (Goller et al. 2004; Green 2007; Harris et al. 2008; Glass et al. 2009).

A alta transmissibilidade, resultante da baixa dose infecciosa (<100 partículas virais) e do alto título viral excretado nas fezes, o prolongado período de excreção viral (até um mês pós infecção), a estabilidade no ambiente, se mantendo infecciosos em superfícies e água contaminadas e a diversidade genética e antigênica, onde repetidas infecções podem ocorrer ao longo da vida facilitam a dispersão dos NoV, tornando-os importantes contaminantes ambientais (Graham et al. 1994; Estes et al. 2006; Atmar et al. 2008; Teunis et al. 2008). Estudos têm demonstrado a importância de se analisar a presença destes vírus em diferentes matrizes ambientais incluindo superfícies, fômites e ecossistemas aquáticos (Berg et al. 2005; Ueki et al. 2005; Gallimore et al. 2006).

Surtos de gastroenterite causados por NoV associados à água contaminada têm sido relatados desde o final da década de 70. Entre 1977 e 2006, Sinclair e colaboradores (2009) revisaram 25 surtos causados pelos NoV por contato com água contaminada, dentre os quais a maior parte causada em piscinas e lagos (84%). Diversos surtos de GA causados por NoV são associados a ingestão de alimentos contaminados por água, ou pela ingestão de frutos do mar (ostras, mexilhões) cultivados em ambientes aquáticos contaminados (Warner et al. 1991; Hafliger et al. 1999; Appleton et al. 2000).

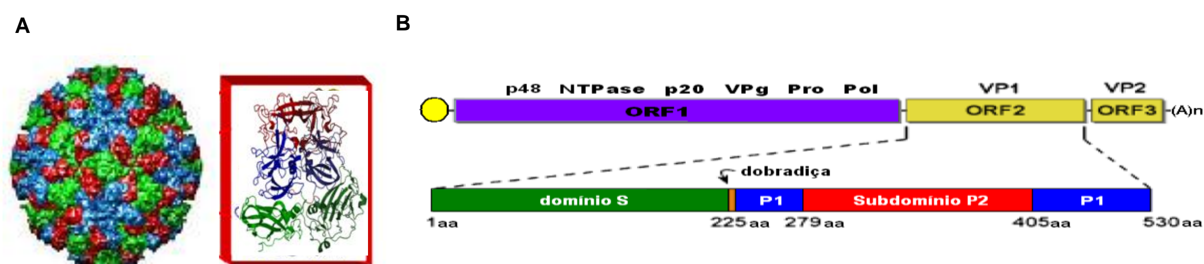

Figura 5: Partícula de norovírus. A) Morfologia. Criomicrografia eletrônica do norovírus murino (MNV-1). Em destaque proteínas do capsídeo viral composta de três subunidades (Sub domínio P1; Subdominio P2; Dominio S. **B) Organização genômica.** p48: proteína amino terminal; NTPase: proteína nucleosídeo trifosfatase; p20: proteína p20; VPg: proteína de união ao genoma; Pro: protease; Pol: RNA polimerase; VP1: proteína principal do capsídeo; VP2: proteína menor do capsídeo; círculo amarelo: VPg; $(A)_n$: Cauda Poli(A); aa: aminoácido. ORF: Fase Aberta de Leitura. [Adaptado de Donaldson et al. (2008)].

1.3.5 Astrovírus

Os astrovírus humanos (HAstV) são classificados na família *Astroviridae*, constituída por dois gêneros, *Mamastrovirus* e *Avastrovirus*. No gênero *Mamastrovirus* encontram-se os AstVs que infectam o homem (HAstV-1 a HAstV-8) e outros mamíferos como bovinos felinos e suínos (Méndez & Arias 2007).

O genoma dos HAstV é constituído por um RNA de fita simples de polaridade positiva com o número de nucleotídeos variando de 6844-7355 (Walter & Mitchell 2003). Contém três ORFs, chamadas ORF-1a, ORF-1b e ORF-2. A ORF-1a e a ORF-1b se encontram na região 5' do genoma e codificam as proteínas não estruturais (NSPs); a ORF-2, localizada na região 3', codifica as proteínas estruturais do capsídeo (Méndez & Arias 2007) (Figura 6).

Os HAstV podem ser classificados segundo o genótipo, com base na seqüência nucleotídica de uma região de 382 pb da segunda fase aberta de leitura (ORF-2) (Noel et al. 1995). Análises filogenéticas demonstraram que os sorotipos de HAstV podem ser classificados em 8 genótipos correlacionados aos sorotipos, sendo que os sorotipos HAstV-4 e 8 foram agrupados como genótipo 4 (Méndez & Arias 2007).

Estudos realizados com voluntários demonstraram que as infecções por HAstV são adquiridas pelo contato pessoa-pessoa e pela ingestão de água e alimentos contaminados, tais como, mariscos e ostras provenientes de áreas poluídas por esgotos (Abad et al. 2001; Mitchell 2002; Gofti-Laroche et al. 2003; Walter & Mitchell 2003).

Crianças de até cinco anos são as mais susceptíveis à infecção, tendo sido descritas epidemias em escolas, creches e unidades pediátricas. Os HAstV também estão associados a surtos de GA em adultos, idosos e pacientes imunodeficientes (Leite et al. 1991; Liste et al. 2000; Sakamoto et al. 2000; Silva et al. 2001; Cardoso et al. 2002; Victoria et al. 2007).

Em países desenvolvidos, a prevalência de gastroenterite associada a HAstV varia de 2 a 11% e nos países em desenvolvimento de 2 a 26% (Cunlife et al. 2002; Dalton et al. 2002; Ratcliff et al. 2002). A prevalência de cada sorotipo também varia segundo a localização geográfica (Sakamoto et al. 2000) e a época do ano (Guix et al. 2002). O HAstV-1 tem sido relatado como o sorotipo predominante em alguns países da Europa, EUA, Austrália, Japão, Coréia do Sul e Brasil (Noel et al. 1995; Liste et al. 2000; Méndez-Toss et al. 2000; Sakamoto et al. 2000; Silva et al. 2001; Cardoso et al. 2002; Dalton et al. 2002; Gabbay et al. 2005). No Brasil, estudos em diferentes cidades demonstram a prevalência do HAstV varia de 2,8% a 14% em crianças hospitalizadas com GA de até cinco anos de idade, correspondendo a

maioria dos casos ao HAstV-1 sendo também detectados os HAstV-2, 3, 4, 5 e 8 (Cardoso et al. 2002; Gabbay et al. 2005; 2006; Silva et al. 2006; Victoria et al. 2007).

Apesar de estudos apontarem os HAstV como importantes agentes etiológicos causadores de GA, a detecção de HAstV em amostras fecais e ambientais não acontece de forma rotineira. Com isso existem poucos relatos da presença de HAstV em ambientes aquáticos ou surtos relacionados. Maunula e colaboradores (2004) associaram os HAstV a um surto de GA em uma piscina na Escandinávia. Alguns estudos demonstram a presença de HAstV em amostras de águas residuárias (Le Cann et al. 2004; Rodríguez-Díaz et al. 2009).

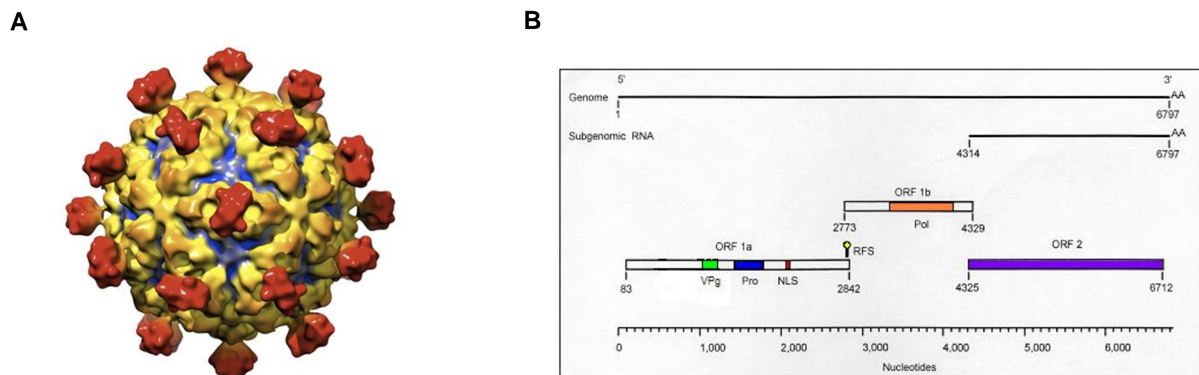

Figura 6: Partícula de astrovírus. A) Criomicroscopia eletrônica. B) Organização do genoma. VPg: Proteína de união ao genoma; Pro: Protease; NLS: Sinal de localização nuclear Pol: Polimerase; RFS: Estrutura do mecanismo de *frameshifting* ribossômico. (Adaptado de Jiang et al, 1993; Matsui & Greenberg, 2001).

2. RELEVÂNCIA

Entre a grande diversidade de vírus que infectam o homem, muitos são excretados em grandes concentrações nas fezes ou urina de pessoas, estando presentes em grandes quantidades em águas residuárias urbanas e considerados contaminantes ambientais. O tratamento atualmente aplicado às águas residuárias processadas por métodos biológicos e físico-químicos tem reduzido significativamente a incidência de enfermidades entre a população, especialmente as de etiologia bacteriana. Entretanto, os vírus são mais resistentes que as bactérias a muitos destes tratamentos. Concentrações significativas de vírus são detectadas em águas vertidas no ambiente e nos biosólidos gerados em plantas de tratamento de água residual (Hurst 1997; Bosch et al. 2008). A contaminação do meio ambiente, a partir de águas residuárias, se confirma pela presença de vírus em águas superficiais de rios e lagos, onde se detectam altos percentuais de amostras positivas (De Paula et al. 2007, Miagostovich et al. 2008) e em frequentes casos de infecções virais associados ao consumo de moluscos bivalves que se observam, a cada ano, em países industrializados (Lees 2000; Sinclair et al. 2008).

Ao contrário da poluição por bactérias e fungos, a presença de partículas virais na água é de difícil detecção e exige tecnologias sofisticadas de concentração desses agentes e, na maioria dos casos, detecção molecular (Metcalf et al. 1995; Wyn-Jones & Sellwood 2001).

O controle virológico do meio ambiente é um processo complexo devido à dificuldade de identificar concentrações normalmente baixas de vírus pertencentes a diferentes famílias e que estão dispersos em grandes volumes de água, biosólidos ou em outro tipo de amostras ambientais. A aplicação da (RT)-PCR tem permitido identificar o papel dos RVA, NoV e HAstV como causadores de GA por veiculação hídrica. Os HAdV e os JCPyV também são identificados entre os vírus mais frequentemente detectados em águas residuárias, tendo sido apontados como potenciais indicadores virais de contaminação humana (Bofill-Mas et al. 2000; Pina et al. 1998; Wyn-Jones & Sellwood 2001; Godoy et al. 2006).

Informações referentes à concentração de agentes virais em águas residuárias têm sido obtidas principalmente em países desenvolvidos (Girones et al. 2010; Okoh et al. 2010). Entretanto, a necessidade de se estabelecer marcadores virais de contaminação fecal humana no ambiente deve ser amparada por dados de disseminação obtidos em diferentes regiões geográficas (Cantalupo et al. 2011). Adicionalmente, a caracterização dos vírus detectados em águas residuárias disponibiliza dados para a realização de estudos epidemiológicos sobre a circulação de determinados genótipos na região estudada.

Neste trabalho, investigou-se a disseminação ambiental dos principais vírus responsáveis pelos quadros de GA (RVA, NoV, HAstV), assim como dos vírus DNA (HAdV e JCPyV), potenciais marcadores virais, em águas residuárias de duas ETEs localizadas na cidade do Rio de Janeiro. Em um primeiro momento, em amostras obtidas de uma ETE piloto (ETE Fiocruz), diferentes metodologias de concentração, detecção e quantificação destes vírus foram estabelecidas e avaliadas, assim como a utilização do bacteriófago PP7 como controle interno. Posteriormente, as metodologias desenvolvidas foram aplicadas em uma ETE de grande porte (ETE Alegria) para avaliação da concentração da carga viral e determinação da diversidade dos genótipos circulantes. A realização dos monitoramentos em diferentes períodos, também permitiu, pela abordagem ambiental, se avaliar o impacto da circulação dos diferentes genótipos de RVA nos anos pré e pós a introdução da vacina anti-rotavírus (Rotarix®) no PNI em março de 2006.

3. OBJETIVOS

3.1 Objetivo geral

- Estabelecer, avaliar e aplicar metodologias de concentração, detecção, quantificação e caracterização molecular de RVA, NoV, HAstV, HAdV e JCPyV em águas residuárias da cidade do Rio de Janeiro, fornecendo dados sobre a disseminação e diversidade dos genótipos virais excretados pela população, assim como avaliar a concentração dos diferentes vírus indicando um marcador virológico de contaminação ambiental.

3.2 Objetivos específicos

1- Avaliar a disseminação de RVA, NoV e HAstV em águas residuárias da ETE-Fiocruz (Rio de Janeiro), determinando a prevalência dos genótipos circulantes.

2- Estabelecer metodologias de detecção, quantificação e caracterização molecular de JCPyV e avaliar a presença destes vírus em águas residuárias da ETE-Fiocruz (Rio de Janeiro).

3- Estabelecer metodologia de quantificação (qPCR) para bacteriófago PP7 a fim de se avaliar sua utilização como controle interno nos métodos de recuperação viral.

4- Estabelecer qPCR para RVA e avaliar esta técnica no formato multiplex para detecção simultânea de RVA e do controle interno PP7.

5- Avaliar o método de ultracentrifugação para concentração de RVA comparando sua eficiência de recuperação com o método de filtração em membrana negativa.

6- Avaliar a presença e concentração de RVA em uma ETE de grande porte (ETE Alegria - CEDAE-RJ), e determinar a diversidade de genótipos e a circulação de cepas vacinais.

7- Determinar a disseminação e concentração viral de NoV, HAstV, HAdV e JCPyV em amostras de afluente e efluente da ETE Alegria a fim de se avaliar um marcador viral de contaminação humana em ecossistemas aquáticos.

4. METODOLOGIAS e RESULTADOS

As seções "Metodologias" e "Resultados" deste trabalho de tese serão apresentados sob forma de manuscritos publicados e submetido à publicação.

Os resultados relacionados aos objetivos específicos 1 e 2 foram desenvolvidos numa ETE de pequeno porte (ETE Fiocruz) e estão apresentados nos manuscritos 4.1; 4.2; 4.3 e 4.4. Os resultados obtidos do estudo realizado na ETE de grande porte (ETE Alegria) estão apresentados nos manuscritos 4.6 e 4.7.

4.1 Molecular detection of human astrovirus in an urban sewage treatment plant in Rio de Janeiro, Brazil

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Molecular detection of human astrovirus in an urban sewage treatment plant in Rio de Janeiro, Brazil

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Objetivos:

- Avaliar a disseminação de HAstV em águas residuárias da ETE-Fiocruz (Rio de Janeiro) e determinar a prevalência dos genótipos circulantes.

Molecular detection of human astrovirus in an urban sewage treatment plant in Rio de Janeiro, Brazil

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The objective of this study was to evaluate the prevalence and dissemination of human astroviruses (HAstV) in the environment by analyzing urban sewage samples from a wastewater treatment plant in the city of Rio de Janeiro, Brazil. A one-year study was performed with a total of 48 raw and treated sewage composite samples, which were collected biweekly from an activated sludge plant. Virus particles were concentrated by the adsorption-elution method using negatively charged membranes associated to a Centriprep Concentrator® 50 (Nihon Millipore). HAstV were detected in 16.7% of the samples in raw and treated sewage by using both qualitative and quantitative reverse transcriptase-polymerase chain reactions (RT-PCR and qPCR, respectively). Positive untreated sewage sample exhibited mean values of 1.1 x 10^4 gEq/mL. The qPCR sensitivity was 18 gEq/reaction. Through utilization of qPCR, a HAstV recovery efficiency of 4.2% and 4.3% was demonstrated for raw and treated sewage samples, respectively. The presence of HAstV in both the raw and treated sewage samples demonstrated the dissemination of these viruses in the environment as well as viral permanence after sewage treatment. There was a reduction in the total and faecal coliform levels, indicating efficiency of the wastewater treatment plant.

Key words: astroviruses - wastewater - RT-PCR - qPCR

Recent developments in improved surveillance, routine screening and the application of sensitive molecular assays have increased recognition of enteric viruses as environmental contminants. Furthermore, the burden of human astroviruses (HAstV) infections has been well reported and recognized as important secondary etiologic agents of viral gastroenteritis (Wilhelmi et al. 2003).

Due to the growing importance of HAstV in cases of acute gastroenteritis among children, studies in Europe, USA, South America and Africa have investigated these viruses in the environment and demonstrated HAstV presence in rivers, reservoirs, residual waters and sludge (Gofti-Laroche et al. 2003, Le Cann et al. 2004, Meleg et al. 2006, Miagostovich et al. 2008).

HAstV are non-enveloped viruses with icosahedral symmetry of 28 nm in diameter, which belong to the *Astroviridae* family. The viral genome consists of a single-stranded, positive sense RNA molecule that is polyadenylated and comprised of approximately 6.8-7.2 kilobases, with three open reading frames (ORFs), designated ORF1a, ORF1b and ORF2 (Jonassen et al. 2003). HAstV are classified into eight genotypes (HAstV-1-HAstV-8), based on the phylogenetic analysis of ORF2. HAstV-1 has been described as the most prevalent genotype worldwide (Noel et al. 1995, Guix et al. 2002, Silva et al. 2006).

The objective of this study was to evaluate the prevalence and dissemination of HAstV in environmental samples using an adsorption-elution method for viral detection by using a negatively charged membrane technique, which was previously described for recovery of enteric viruses from seawater (Katayama et al. 2002). For this purpose, a one-year study was performed in an urban wastewater treatment plant in the city of Rio de Janeiro, since residual waters are the main source of pathogenic microorganisms. Therefore, such environments provide information regarding the different strains infecting human populations. Total and faecal coliforms were also investigated to characterize faecal contamination in the samples. To our knowledge, this is the first study demonstrating the circulation of HAstV in sewage samples of Brazil.

MATERIAL AND METHODS

Sewage samples - From January-December of 2005, 48 raw and treated sewage composite samples were collected biweekly from an activated sludge plant in the city of Rio de Janeiro, Brazil. Eight 250 mL aliquots were collected for each sample and a total of 2 L samples were stored in glass bottles. The samples were taken to the laboratory and immediately analysed for bacterial parameters. The samples were processed for viral concentration in the following 24 h and stored at -80°C, until utilized for virus detection assays. Samples from influent and effluent were collected as positive and negative control.

Bacterial parameters - Total coliform (TC) and faecal coliform (FC) were measured using the Colilert®-18

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Quanti-Tray®/2000 method (IDEXX Laboratories, Westbrook, USA).

Viral particle concentration method - HAstV were concentrated using an adsorption-elution method with negatively charged membranes, which included the insertion of an acid rinse step for removal of cations, as previously described (Katayama et al. 2002). Prior to process filtration, 1.2 $MgCl_2$ was added in 2 L of water. The system was soaked briefly in a 10% bleach solution and rinsed in distilled water prior to each use. The eluate (10 mL) was re-concentrated to a final volume of 2 mL using a Centriprep Concentrator® 50 (Nihon Millipore).

Recovery efficiency of the method for HAstV concentration - In order to evaluate the recovery efficiency of HAstV from raw and untreated sewage, 100 µL of the 10% fecal suspension of HAstV genotype 1 strain (GenBank accession number DQ381498), prepared in Tris/HCl/Ca^{++} 0.01M pH 7.2 buffer, was spiked with HAstV. The negative control, without HAstV spiking, was also tested in order to certify the absence of a natural contamination. All assays for viral concentration were performed in triplicate (independent experiments) for both treated and untreated wastewater.

Extraction of viral RNA - Prior to extraction, 70 µL of Vertrel® (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) was added to 140 µL of the sample and then centrifuged at 800 *g* for 10 min. Nucleic acid extraction from the supernatant was performed using a QIAmp Viral RNA Mini Kit® (Qiagen, Inc, Valencia, California, USA), following the manufacturer's protocol.

Reverse transcription reaction (RT) - The synthesis of cDNA was performed with RT using a random primer (PdN6, 50 A260 units, Amersham Biosciences, Chalfont St Giles, Buckinghamshire, UK) as previously described (Ferreira et al. 2008).

Enzymatic amplification - (i) Reverse transcription-Polymerase chain reaction (RT-PCR). Amplification was performed using 10-µL aliquots of the cDNA, as described in a previous study (Noel et al 1995). HAstV type 2 strain, obtained from faecal suspension at the Regional Reference Centre of Rotaviruses, was used as positive control. To exclude the possibility of cross-contamination, all reagents used for PCR were prepared in a laminar flow cabinet. A positive and a negative control were included in all PCR reactions. (ii) Quantitative Real-time PCR (qPCR). For specific detection and quantification of the HAstV genome, 5 µL of cDNA was also assayed. Amplification was performed in a 25 µL reaction mixture with the PCR Master Mix® (Applied Biosystems, Branchbug, New Jersey, USA). The reaction contained 5 µL of a cDNA sample or 10 µL of a quantified plasmid DNA, 1X TaqMan master mix, and the corresponding primers and TaqMan probes at the appropriate concentrations. HAstV genomes were quantified with 0.1 µM of the primers AV1 and AV2 and 0.15 µM of the fluorogenic probe AVS, as described by LeCann et al. (2004). Then, the uracil N-glycosilase in the core mix was activated (2 min at 50°C), followed by activation of the AmpliTaq Gold for 10 min at 95°C and 45 cycles (15 s at 94°C and 1 min at 55°C), performed with an ABI 7500® (Applied Biosystem, California, USA). All samples were performed in duplicate. Positive and negative controls were included. The amount of DNA was defined as the average of the duplicate data obtained. The RT-PCR/qPCR reactions were performed in separate rooms from those used for viral isolation and processing of water samples. Table I shows the sequences of the primers used in both enzymatic amplification reactions.

TABLE I

Primers and probe used for amplification and detection of human astrovirus

Name	Nucleotide sequence (5' -3')
Mon 269 (+)[a]	^{4526}CAA CTC AGG AAA CAG GGT GT^{4545}
Mon 270 (-)[a]	^{4955}TCA GAT GCA TTG TCA TTG GT^{4974}
AV1 (+)[b]	^{6775}CCG AGT AGG ATC GAG GGT^{6797}
AV2 (-)[b]	^{6708}GCT TCT GAT TAA ATC AAT TTT AA^{6725}
AVS (Probe)[b]	$^{6739\ Fam-}$CTT TTC TGT CTC TGT TTA GAT TA T TTT AAT CAC C$^{-Tamra\ 6772}$

a: primers used for qualitative amplification (Noel et al. 1995); *b*: primers and probe used for quantitative polymerase chain reaction (Le Cann et al. 2004).

RESULTS

Detection and quantification of HAstV - The RT-PCR and qPCR protocol were both applied to 48 sewage samples: 24 from inflow and 24 from outflow. A total of seven HAstV strains (14.6%) were detectable by using qualitative RT-PCR, while qPCR detected one positive sample (2.1%) in a raw sewage with a titre of 1.1 x 10^4 G eq/mL. By using both methodologies, the total number of positive samples was determined to be 16.7% (8/48) of HAstV detected. The detection limit of the qPCR was 18 gEq/reaction. The standard curve demonstrated a correlation coefficient (R2) from 0.995-0.999 and slope varying from -3.64-4.0. The HAstV recovery efficiency was 4.2% and 4.3% for raw and treated sewage samples, respectively, as demonstrated by qPCR.

After sedimentation and biological secondary treatment (activated sludge), 99.9% of TC and 99.9% of FC were removed (Table II). The efficiency of HAstV removal could not be calculated, since positive samples could not be quantified from the raw sewage. HAstV were detected in both outflow and inflow water samples collected on the same day (Table II).

DISCUSSION

In Brazil, studies have been developed in order to evaluate the microbiological quality of water, with an emphasis on bacterial contamination (Alves et al. 2002, Nogueira et al. 2003). However, few studies have investigated the presence of human enteric viruses in water samples (Mehnert & Stewien 1993, Mehnert et al. 1997, Villar et al. 2006, Miagostovich et al. 2008). The ab-

TABLE II

Microbiological results obtained from water samples collected at sewage treatment plant in Rio de Janeiro, 2005

Samples		Raw sewage			Treated sewage			Removal efficiency	
Month	Sampling collection	Total coliform	Faecal coliform	HAstV detection	Total coliform	Faecal coliform	HAstV detection	Total coliform %	Faecal coliform %
Jan	1	1,90E+06	6,20E+05	-	5,45E+04	4,10E+03	+	97.13	99.99
	2	5,30E+06	1,10E+06	-	6,60E+04	1,00E+04	-	98.75	99.99
Feb	3	ND	ND	-	ND	ND	+	ND	ND
	4	2,31E+07	7,40E+06	-	3,69E+05	1,34E+05	-	98.40	99.99
Mar	5	5,94E+07	8,60E+06	-	3,73E+05	5,20E+04	-	99.37	99.99
	6	2,31E+07	7,40E+06	-	6,97E+04	1,10E+04	-	99.99	99.99
Apr	7	6,80E+07	7,10E+06	-	1,90E+05	2,40E+04	-	99.72	99.99
	8	7,91E+09	2,43E+08	-	1,19E+05	2,00E+04	-	99.99	99.99
May	9	5,25E+08	3,05E+07	-	3,93E+04	2,00E+03	-	99.99	99.99
	10	ND	ND	-	ND	ND	-	ND	ND
Jun	11	ND	ND	-	ND	ND	-	ND	ND
	12	1,00E+08	1,33E+07	-	5,73E+05	7,40E+04	-	99.43	99.99
Jul	13	1,18E+08	4,86E+07	-	1,03E+05	1,43E+04	-	99.91	99.99
	14	1,78E+09	5,20E+07	+	1,66E+06	1,20E+05	+	99.91	99.99
Aug	15	1,45E+09	1,00E+07	-	6,60E+05	2,70E+04	-	99.95	99.99
	16	5,24E+08	7,40E+06	-	3,28E+05	8,50E+03	-	99.94	99.99
Sep	17	8,29E+09	3,10E+07	+	9,30E+06	ND	-	99.88	ND
	18	3,13E+09	3,00E+07	+	1,90E+06	2,00E+04	-	99.94	99.93
Oct	19	7,27E+09	1,34E+08	-	1,12E+07	8,60E+04	-	99.85	99.99
	20	1,39E+09	5,30E+07	+[a]	1,20E+06	4,10E+04	+	99.91	99.99
Nov	21	1,57E+08	2,28E+07	-	3,13E+05	1,00E+04	-	99.80	99.99
	22	6,21E+08	1,65E+08	-	2,39E+05	9,10E+04	-	99.96	99.99
Dec	23	4,65E+08	4,00E+07	-	2,22E+04	1,00E+04	-	99.99	99.99
	24	2,28E+08	1,00E+07	-	6,24E+04	9,70E+04	-	99.97	99.99

a: HAstV detection by qPCR; ND: not done.

sence of viral concentration methods of high recuperation efficiency and detection methods of low cost have been indicated as the primary reason for the low number of studies in the area of environmental virology. There is a growing demand for studies that establish fast and sensitive methods for the detection of viruses in environmental samples.

The recovery efficiency of the virus concentration method based on electrostatic interactions among viruses and an electronegative filter was previously evaluated for poliovirus (Katayama et al. 2002). Recently, the recovery of noroviruses and sapoviruses from sewage treatment plant also demonstrated the efficiency of this method for concentration and detection of viruses in water samples (Haramoto et al. 2006, 2008). In this study, the recovery of HAstV in both influent and effluent of the wastewater treatment plant was demonstrated using this methodology. However, the prevalence of HAstV (16.7%) was low when compared with other studies, with high indexes ranging from 43-100% and 82.3% from inflow and outflow wastewater samples, respectively (Nadan et al. 2003, Le Cann et al. 2004, Meleg et al. 2006). The high percentage of HAstV detected in sewage samples are usually explained due to the high seroprevalence (80-90%) of HAstV in the studied population, the high stability of these viruses in the environment, and the efficiency of the ultracentrifugation method that is traditionally used to concentrate and recover viral particles from wastewater (Le Cann et al. 2004). The low recovery of HAstV could be due to the low efficiency of the method used in this study and/or the low organic load supplied to the sewage treatment plant.

The prevalence of gastroenteritis associated with HAstV infection ranges from 2-11% and 2-26% in the developed and developing countries, respectively (Chikhin-Brachet et al. 2002, Cunlife et al. 2002, Dalton et al. 2002, Ratcliff et al. 2002). In Brazil, the prevalence of HAstV in the paediatric population ranged from 2-28% (Cardoso et al. 2002, Gabbay et al. 2005, Silva et al. 2006, Resque et al. 2007, Victoria et al. 2007, Soares et al. 2008). The Regional Reference Centre of Rotaviruses demonstrated a 7.8% prevalence of HAstV infection in the city of Rio de Janeiro in 2005 (unpublished data), which was lower than the prevalence detected from environmental samples in this study. According to studies based on HAstV detection in environmental samples, asymptomatic and/or mild digestive morbidity HAstV infections could create an underestimation of the real prevalence of infection by this virus in the population (Meleg et al. 2006). Studies with environmental

samples have been suggested to replace those with clinical samples in order better determine the circulation of HAstV, since there is a higher prevalence of this virus in the environment than in clinical samples.

The presence of HAstV in both raw and treated samples demonstrates the resistance of these viruses to wastewater treatment and corroborates previous studies that also detected HAstV in inflow and outflow waters (Nadan et al. 2003, Le Cann et al. 2004, Meleg et al. 2006). This data suggest that HAstV resist sewage treatment at the activated sludge plant, remaining in the environment after being discharged into water body and is not related to the observed high removal efficiency of the total and faecal coliforms. Data obtained with these samples were previously published and demonstrated that the removal index of hepatitis A virus HAV (42.3%) was less than that for TC and FC (Villar et al. 2006). Molecular methods used for detecting HAstV cannot distinguish between infectious and non-infectious virions, although they provide a rapid and sensitive method to detect viruses as an alternative to overcome the limitations of conventional techniques, such as cell cultures, since HAstV are considered fastidious viruses. The detection of a single strand RNA genome in the environment has suggested the presence of infective viruses, since this molecule is not very stable in the environment (Meleg et al. 2006).

Although described as a more sensitive method that is recommended for investigation of environmental samples with low viral concentrations (Laverick et al. 2004), utilization of qPCR (Le Cann et al. 2004) did not present satisfactory results. Previous investigations have indicated lower sensitivity of the qPCR technique in comparison to traditional PCR (Noble et al. 2003, Grimm et al. 2004, Bastien et al. 2008).

To our knowledge, this is the first study demonstrating the detection of HAstV in a sewage treatment plant in Brazil. In a previous study, this methodology demonstrated the recovery of many human enteric viruses in the Amazon basin, with a 15.4% prevalence of HAstV in river waters (Miagostovich et al. 2008). This methodology, designed to both concentrate and detect HAstV in environmental samples, offers a new perspective for evaluation of viral circulation amongst the population via environmental dissemination.

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4.2 Environmental dissemination of group A rotavirus: P-type, G-type and subgroup characterization

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Environmental dissemination of group A rotavirus: P-type, G-type and subgroup characterization

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Objetivos:

- Avaliar a disseminação de RVA em águas residuárias da ETE-Fiocruz (Rio de Janeiro) e determinar a prevalência dos genótipos circulantes.

Environmental dissemination of group A rotavirus: P-type, G-type and subgroup characterization

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ABSTRACT

Rotaviruses A (RV-A) infection is the most common cause of acute diarrheal diseases in infants and the dissemination of these viruses in the environment represents a public health hazard. The present study aims to evaluate reverse transcription-polymerase chain reaction (RT-PCR) based protocols for the detection of RV-A genes in different types of environmental samples. RV-A were concentrated by the adsorption-elution method using negatively charged membranes associated with a Centriprep Concentrator 50. The RV-A VP4, VP7 and VP6 genes were detected using RT-PCR in river water from the Amazon Hydrographic basin (Northern region) and from wastewater in a sewage treatment plant in Rio de Janeiro (Southeast region), Brazil. RV-A were successfully detected in water environmental samples by the methods used. The detection of the VP6 gene by RT-PCR was the most sensitive for detecting RV-A in environmental samples (44.0%), when compared to the detection of the VP4 (33.3%) and VP7 (25.3%) genes. Based on nucleotide sequence and phylogenetic analysis of the partial VP6 gene, 22 environmental samples were determined to be subgroup II (Wa-like). These results indicate that analysis of environmental samples could possibly make a valuable contribution to studies on the epidemiology of RV-A.

Key words | river water, Rotavirus A, VP4, VP6, VP7, wastewater

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INTRODUCTION

Rotavirus infection is the most common cause of acute diarrheal diseases in infants and young children worldwide and in developing countries is responsible for one third of all diarrhea-associated hospitalization (Parashar *et al.* 2006)

Rotavirus is a genus of the *Reoviridae* family and the virion is a non-enveloped particle consisting of a triple-layered capsid structure that surrounds the genome of 11 segments of double-stranded RNA (dsRNA). Six structural proteins (VP1, 2, 3, 4, 6 and 7) and six nonstructural proteins (NSP1 to NSP6) are encoded by a unique genome segment except for NSP5 and NSP6, which are encoded by an overlapping reading frame of gene 11. Rotaviruses can be classified into groups (A to G). Group A rotaviruses (RV-A) are the most prevalent of the seven groups and can be differentiated into four subgroups (I; II; I and II or not I, not II) based on the antigenic properties of the VP6 protein (middle capsid layer) (Villena *et al.* 2003; Parashar *et al.* 2006). On the basis of the VP4 and VP7 genes, which encode the two outer capsid proteins, RV-A can be classified into at least 27 P genotypes and 19 G genotypes (Khamrin *et al.* 2007; Martella *et al.* 2007; Matthijnssens *et al.* 2008).

RV-A are excreted in high concentrations in feces and their stability in aqueous environments and their resistance to water treatments may facilitate their transmission to humans (Ansari *et al.* 1991; Espinosa *et al.* 2008). Although it

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is difficult to determine the proportion of diarrhea cases due to waterborne transmission, it has been suggested that a significant proportion of the cases is related to water quality (Bosch *et al.* 2008).

During the past decade, reverse transcription-polymerase chain reaction (RT-PCR) has emerged as an efficient method to detect enteric viruses, including RV-A in water samples (Baggi & Peduzzi 2000; Pusch *et al.* 2005). The present study aims to evaluate different RT-PCR protocols for the amplification of the VP4, VP7 and VP6 genes in order to select a convenient method for examining the environment dissemination of RV-A. Additionally, VP4 and VP7 RT-PCR results and a partial VP6 nucleotide sequencing will provide information about the genotypes circulating in two regions of Brazil (Northern and Southeast) by recovering viruses from river water and wastewater samples.

METHODS

River water samples

Fifty-two river water samples were obtained from a dense hydrographic network composed of microbasins, such as the Tarumã-Açu, São Raimundo, Educandos and Puraquequara, that surrounds the city of Manaus in the State of Amazonas, Northern region of Brazil. The 1-year environmental surveillance was based on four sample collections at each site, conducted between August 2004 and June 2005 as previously described (Miagostovich *et al.* 2008). The São Raimundo and Educandos basins are characterized as urban areas with different levels of water degradation process, which is caused mainly by the complete or partial removal of riparian vegetation and pollution with domestic sewage. Two-liter samples of surface river water were collected in sterile bottles, transferred to the laboratory and stored at 4°C.

Sewage samples

A total of 48 raw and treated sewage composite samples were obtained biweekly (2005) from an activated sludge plant in Rio de Janeiro, Southeast region of Brazil. Each 2 L composite sample consisted of eight 250 mL grab samples collected at regular intervals and stored in a glass bottle at 4 °C.

Concentration of viruses from water and viral RNA extraction

Both types of water samples were concentrated using a negatively charged membrane which included the insertion of an acid rinse step for removal of cations, following the methodology described by Katayama *et al.* (2002). Briefly, $MgCl_2$ was added to every sample to a final concentration of 3 mM, passed through a fiber glass membrane AP20 (Nihon Millipore®, Tokyo, Japan) to eliminate large-particulate, then through an HA (mixed cellulose esters) negatively charged membrane filter (0.45 mm pore size and 142 mm diameter Nihon Millipore®, Tokyo, Japan). The filter was rinsed with 350 mL of H_2SO_4 (0.5 mM, pH 3.0) and the viruses were eluted with 15 mL of NaOH (1 mM, pH 10.8). The filtrate was recovered in a tube containing 50 μL of H_2SO_4 (100 mM, pH 1.0) and 50 μL of 100 × Tris–EDTA buffer (pH 8.0) for pH neutralization, followed by centrifugation using a Centriprep YM-50®, (Millipore) at 1,500 g for 10 min to obtain a final concentrate volume of 2 mL.

A 2 mL aliquot of the concentrated sample was processed for nucleic acid extraction using the Viral RNA Mini QIAamp Kit® (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions and the RNA was stored at −70°C. Sewage samples were clarified by adding 70 μL of Vertrel1® (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) and centrifuged at 800 × g for 10 min before RNA extraction.

RT-PCR

Briefly, 1 μL of dimethylsulfoxide and 5 μl of RNA were mixed, heated at 97°C for 7 min and chilled on ice for 2 min. Each RT reaction (25 μL final volume) contained: 2.5 mM of each deoxynucleoside triphosphate (Invitrogen, Carlsbad, CA, USA), 1.5 mM MgCl2, 100 U of Superscript II reverse transcriptase (Invitrogen) and 1 μl of random primer (PdN_6–50 A_{260} units - Amersham Biosciences, Chalfont St Giles, Buckinghamshire, UK). The RT reaction mixture was incubated at 42°C for 60 min and at 95°C for

10 min. The PCR primers and protocols for the detection of the VP4, VP7 and VP6 genes have been previously described (Gentsch *et al.* 1992; Das *et al.* 1994; Gouvea *et al.* 1994; Iturriza-Gómara *et al.* 2002). The RT-PCR reactions were prepared in laminar flow cabinets in four separated rooms. All reactions were processed in a thermal cycler (PTC-100 Programmable Thermal Controller, MJ Research, Watertown, MA, USA). To avoid false-positive results, quality control measures were followed as recommended and for each set of amplifications, negative and positive control samples were included. A previously characterized RV-A strains obtained from fecal samples were used as positive control.

Sequencing of the VP6 RT- PCR products

The VP6 amplicons obtained from RV-A positive water samples were purified using a Gel Extraction QIAquick Kit® (QIAGEN), following the manufacturer's recommendations. The purified DNA amplicons were sequenced using an ABI Prism® 3100 Genetic Analyzer and the Big Dye® Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, CA, USA). Centri-Sep columns (Princeton Separations, CA, USA) were used for the purification of the sequencing reaction products, according to the manufacturer's recommendation. The BioEdit Sequence Alignment Editor Program was used to edit and align the sequences obtained (Hall 1999). Genetic analysis was performed using the neighbor-joining method with 1,000 bootstrap replicates and the genetic distance was calculated using Kimura's two-parameter matrix. A phylogenetic tree was constructed using the MEGA v.3.1 software (Kumar *et al.* 2004).

RESULTS AND DISCUSSION

Environmental virology research is still uncommon in developing countries, including Brazil, and little is known about the dissemination of human gastroenteric viruses in Brazilian environmental waters. A few studies have shown the presence of RV-A in sewage, creek water and river water (Mehnert & Stewien 1993; Mehnert *et al.* 1997; Miagostovich *et al.* 2008). The dissemination of these viruses in the environment should be considered as an important route of contamination, mainly in developing countries where a high prevalence of these viruses has been reported (Castello *et al.* 2004; Kane *et al.* 2004; Carvalho-Costa *et al.* 2006). In Brazil 832,762 cases of gastroenteritis due to RV-A infection occurs annually, with 2,475 deaths (Constenla *et al.* 2008).

RV-A VP4 and VP7 genes have been used for the binary molecular classification of RV-A into the G (VP7) and P (VP4) gene types. In this study, a comparative analysis using RT-PCR for the detection of the VP4, VP7 and VP6 genes was performed. Table 1 shows the RT-PCR results obtained using the VP4, VP7 and VP6 genes detection protocols on samples from river water. The VP4 and VP7 results from these samples have been previously published (Miagostovich *et al.* 2008).

The presence of RV-A in river water has been investigated elsewhere and varied from 3.0% to 20.0% in samples from France and Thailand, respectively (Hot *et al.* 2003; Kittigul *et al.* 2005). Although the methodology used in all of the studies should be considered, the higher frequency of RV-A (48.0% by VP6 PCR detection) in river water from Manaus suggests a significant viral contamination of these environments, as a result of domestic sewage discharge without treatment in the streams and inappropriate sanitary conditions. In the Northern region of Brazil, between 1998 and 2000 the city of Manaus reported a 90.5% increase in the number of cases of diarrhea from 8,878 cases to 16,914 (Projeto Geo Manaus 2002). In comparison with the others viruses (adenovirus, astrovirus, norovirus) investigated in the same samples, RV-A was the most abundant gastroenteric virus detected in the river water of the hydrographic basin of Manaus with a positive detection rate ranging from 21% to 48%, depending on the detection method used (Miagostovich *et al.* 2008).

Table 2 shows the RT-PCR results obtained using the VP4, VP7 and VP6 protocols on the wastewater samples from a sewage treatment plant (STP) in Rio de Janeiro. For the sewage samples 45.8% (22/48) were positive for at least one of the genes studied, 11 of which were from raw sewage and 11 were from treated sewage. The genotyping of the RV-A VP4 gene showed three positive samples for P[8], three for P[6] and eleven samples were untypeable. Although the analyses were performed using standardized well established methods for RV-A detection the untypeable

Table 1 | Rotavirus A detection in Manaus based on the detection of the VP4, VP7 and VP6 genes and microbasin investigated. The results from the VP4 and VP7 gene detection have been published previously (Miagostovich *et al.* 2008)

		RV-A RT-PCR					
		VP4		VP7		VP6	
Human settlement level/area type	**Basin (stream)**	**N positive/n. studied (%)**	**Genotype**	**N positive/n. studied (%)**	**Genotype**	**N positive/n. studied (%)**	**N. strains sequenced/SG**
Low/Rural area	Tarumã-Açu (E)	1/4	P[8]	1/4	G1	4/4	AM21/II
	Tarumã-Açu (M)	1/4	P[8]	1/4	G	3/4	–
High/Urban area	São Raimundo (E)	2/4	P[4] P[8]	3\4	G1	1/4	**AM22/II***
	São Raimundo (M)	2/4	P[8]	1/4	G1	4/4	AM40/II
	São Raimundo (M)	2/4	P[8]	1/4	G1	0/4	AM18/II
	São Raimundo (M)	0/4	–	2/4	G1	1/4	AM17/II
High/Urban area	Educandos (E)	3/4	P[8]	1/4	G1	2/4	**AM46/II***
	Educandos (M)	2/4	P[8]	0/4	–	2/4	–
Low/Rural area	Puraquequara (D)	1/4	P[8]	0/4	–	2/4	**AM25/II***
	Tarumã-Açú (D)	0/4	–	0/4	–	2/4	–
Very low/Primary forest	Puraquequara (FR)	0/4	–	1/4	G1	2/4	**AM45/II***
	Tarumã-Açú (FR)	2/4	–	0/4	–	0/4	**AM49/II***
	Tarumã-Açú (FR)	0/4	–	0/4	–	2/4	**AM55/II***
		16/52 (30.8)		11/52 (21.2%)		25/52 (48.0%)	

E = estuary; M = medium; FR = Forest Reserve; D = Decamped area; SG: subgroup; G = not typed.
*Sequences in bold present 100% of nucleotide identity.

Table 2 | Detection and molecular characterization of RV-A based on the VP4, VP7 and VP6 genes in water samples collected at wastewater treatment plant in Rio de Janeiro, 2005

		Raw sewage			Treated sewage		
Month and collecting samples		**VP4 genotype**	**VP7 genotype**	**VP6 Strains/SG**	**VP4 genotype**	**VP7 genotype**	**VP6 Strains/SG**
Jan	1	P	G9	RJ31/II	P	–	–
	2	P	–	–	P	–	–
Feb	3	–	–	–	–	–	–
	4	–	–	–	–	–	+ (ns)*
Mar	5	–	–	–	P	–	–
	6	–	G3	+ (ns)	P	–	–
Apr	7	P	–	–	P[6]	–	+ (ns)
	8	–	–	+ (ns)	–	G4	–
May	9	–	–	–	–	–	–
	10	–	G9	–	–	–	–
Jun	11	–	–	–	–	G1	RJ71/II
	12	–	–	+ (ns)	–	–	–
Jul	13	Nd	Nd	–	–	–	RJ91/II
	14	–	G	RJ92/II	–	G	+ (ns)
Aug	15	–	G	–	P	G1	–
	16	–	G	–	–	–	–
Sep	17	P	–	–	–	–	–
	18	–	–	RJ105/II	P	G1	RJ106/II
Oct	19	–	–	RJ107/II	–	–	+ (ns)
	20	–	G	RJ109/II	P[6]	–	–
Nov	21	P[8]	–	–	P[6]	–	–
	22	P[8]	G	–	–	–	RJ114/II
Dec	23	P[8]	–	RJ115/II	P	–	RJ116/II
	24	–	G	RJ117/II	–	–	–
Total		7/23 (30.4%)	9/23 (39.1%)	10/24 (41.7%)	10/24 (41.7%)	5/24 (20.8%)	9/24 (37.5%)

Nd: Not done; ns: Not sequenced; SG: subgroup; (–): negative; (+): positive; G = not typed; P = not typed.
*RJ: Rio de Janeiro.

strains could be explained by the mismatched with the primers used. Solberg *et al.* (2009) reported novel genotypes that mismatched with most published primers sequences. Then the use of alternate primers should be considered to increase the percentage of RV-A characterization. The typed samples were detected in the first PCR step, using primers for RV-A. In terms of VP7, three samples were characterized as G1, two as G9, one as G3, one as G4 and seven samples were untypeable. Data obtained from the Regional Reference Center of Rotaviruses on the infantile gastroenteritis cases that occurred in the metropolitan area of Rio de Janeiro demonstrated the occurrence of the VP7 genotypes G9, G3, G1, G5, and G2 and the VP4 genotypes P[8], P[4] and P[6]. The genotypes G9P[8], G1P[8], G3P[8] and G2P[4] represent the most frequent RV-A strains circulating in the state of Rio de Janeiro in 2005. It is interesting to emphasize that these genotypes were obtained in a period preceding the introduction of a RV vaccine in the country. The recently developed live and attenuated G1P[8] RV-A vaccine Rotarix (GlaxoSmithKline, Rixensart, Belgium) was introduced into the Brazilian Expanded Immunization Program in March 2006 (Leite *et al.* 2008).

Treatments commonly applied in the STPs have significantly reduced the incidence of bacterial agents in finished water, but viral pathogens can persist following the

treatment process (Bofill-Mas *et al.* 2006). The treatment of this specific STP includes secondary aerobic digestion by activated sludge, extended aeration with biological nitrification and denitrification, without chorination. RV-A was detected in the environment after the sewage treatment (20.8 to 41.7%) with percentages similar to those seen in raw sewage (30.4 to 41.7%). This fact could be explained by the inhibitory factors present in more quantity in the untreated than in treated water samples. It has been demonstrated that the presence of organic compounds such as humic, fulvic, and tannic acids, proteins, and inorganic compounds such as metals present in the environment is a major obstacle to the routine detection of enteric viruses from environmental waters by PCR (Ijzerman *et al.* 1997). Unfortunately, the presence of compounds that could inhibit RT-PCR/PCR was not evaluated. Previous study conducting with the same samples demonstrated a removal index of 42.3% for hepatitis A virus (Villar *et al.* 2006). The efficiency of this STP was demonstrated by the reduction in the total and fecal coliform level, as published elsewhere (Villar *et al.* 2006; Guimarães *et al.* 2008).

Unfortunately, in the present study we could not evaluate the efficiency virus concentration method by negatively charged membrane filtration fro rotavirus detection since we did not have a quantitative method. However our group demonstrated the recovery efficiency for astrovirus in river water (43%), raw sewage (4.2%) and treated sewage (4.3%) (Guimarães *et al.* 2008). Previously, this method showed average recovery yields of spiked poliovirus of 62% from 1 liter of artificial seawater (Katayama *et al.* 2002).

The detection of ssRNA, double-stranded RNA, and DNA viruses by using this methodology in these same environmental samples revealed that the association of the method described here is a feasible approach for detecting enteric viruses in environmental water samples collected from different grades of pollution. The presence of at least one virus in each studied sample (data published elsewhere) validated our results on RV-A detection (De Paula *et al.* 2007; Diniz-Mendes *et al.* 2008; Guimarães *et al.* 2008; Miagostovich *et al.* 2008).

The resistance of RV-A to the active sludge treatment corroborates previous studies that also detected enteric viruses, including astrovirus, norovirus and sapovirus, in inflow and outflow samples using different kinds of treatment, including chlorination (Nadan *et al.* 2003; Le Cann *et al.* 2004; Meleg *et al.* 2006; Haramoto *et al.* 2006; Katayama *et al.* 2008). Although RT-PCR-based detection is a rapid and sensitive tool for the detection of RV-A, it has its limitations since it does not distinguish between infectious and noninfectious viral particles. However, the presence of viral genomes in water samples can be of great value as an indicator of recent viral contamination because of the low stability of free nucleic acids in aqueous environments, especially RNAs (Carducci *et al.* 2003).

The comparative analysis of data obtained from river water and the raw and treated sewage demonstrated that the detection of VP6 was the most efficient for RV-A detection in these environmental samples. Overall, 33.3, 25.3 and 44.0% of the total samples were positive for VP4, VP7 and VP6, respectively. As previously described, the RV-A VP6 gene has been a common target for broadly reactive assays since its detection has a higher sensitivity and specificity (Iturriza-Gómara *et al.* 2002). The high sensitivity of the nested PCR has been described and for RV-A could detect double-stranded RNA from as few as 10 to 100 particles (Gentsch *et al.* 1992).

In order to determine the molecular subgrouping of the RV-A samples twenty-two sequences, twelve from sewage (RJ) and ten from river water (AM), were characterized by sequencing the 379 bp fragment generated from the partial amplification of the VP6 gene and compared to human and animal prototypes of different subgroups, as well as strains obtained from human stool specimens, available in GenBank/NCBI. The nucleotide sequences from strains obtained in this study were deposited in Genbank/NCBI under the accession numbers DQ995863 to DQ995872 (river water) and FJ18704 to FJ18715 (waste water). All RV-A strains were determined to be SG II and clustered with human strains (Figure 1). The RV-A strains obtained from river water samples presented a nucleotide identity ranging from 100% (AM 22, AM25, AM45, AM46, AM49 and AM55) to 89.2%. The strains obtained from the STP in the Southern region of the country had a nucleotide identity that ranged from 100% (RJ31, RJ91, RJ92, RJ105 and RJ106) to 76.3%.

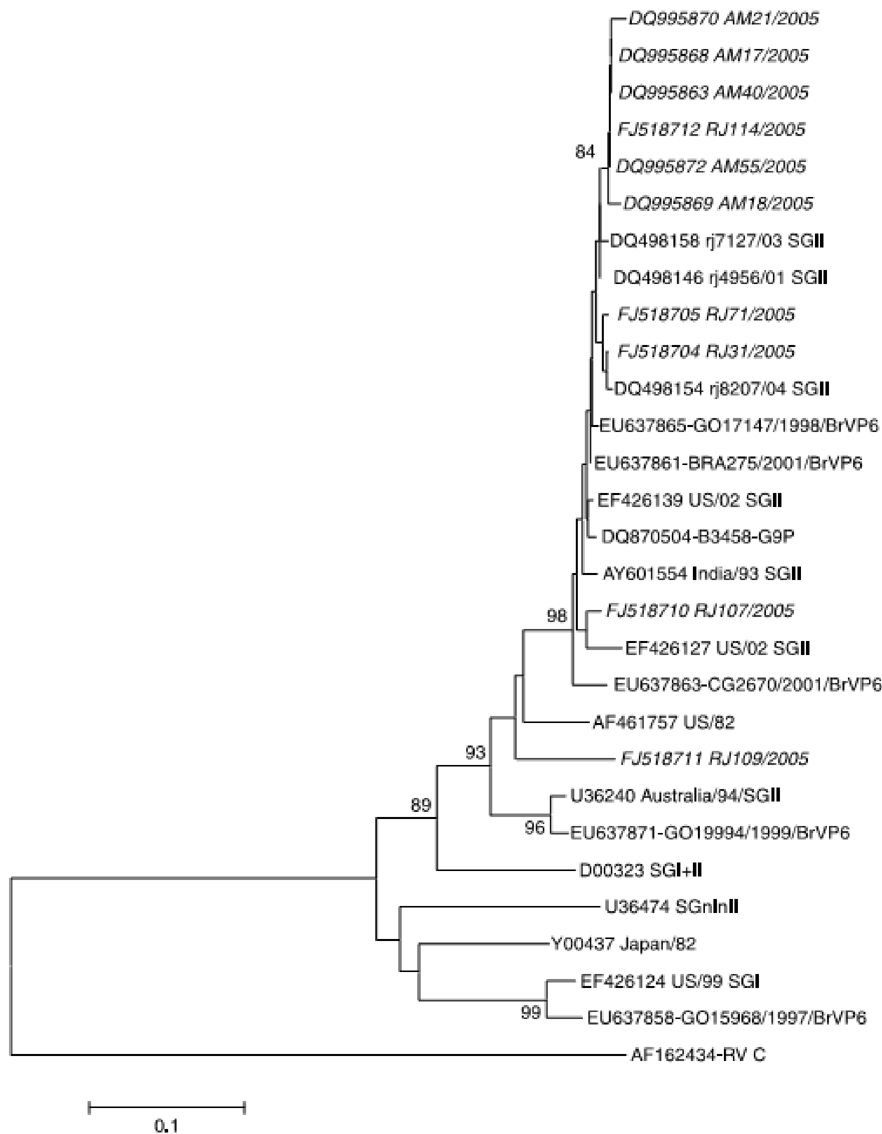

Figure 1 | Phylogenetic tree constructed from the nucleotide sequences of group A rotavirus VP6 genes (nt 747 to 1126). Outgroup: Rotavirus group C (RV C); SG: subgroup; Strain denomination: GenBank (NCBI) accession number. Nucleotide sequences for strains obtained in this study are in bold. AM strains: river samples. RJ strains: sewage samples. Bootstrap values above 70% are indicated.

Among sewage and river samples the nucleotide identity ranged from 100% to 67.8%. Many epidemiological studies have used subgrouping enzyme immunoassays and most of the human isolates fall into SG II, whereas animal isolates fall into SG I (Arista *et al.* 1990; Tang *et al.* 1997; Iturriza-Gómara *et al.* 2002). In Brazil, few studies have characterized the VP6 gene (Araújo *et al.* 2007; Tavares *et al.* 2008). Previously Kerin *et al.* (2007) described a great diversity of VP6 gene. However, in this study the fact that in both areas sequences exhibit 100% of nucleotide identity suggest that those viruses could be more prevalent and then excreted in higher numbers in those populations.

CONCLUSION

The results obtained from the comparison of different RT-PCR protocols for the detection of RV-A demonstrated the VP6 gene is the most suitable for the detection of RV-A in environmental samples. Viral genome detection in environmental samples could contribute to characterizing the RV-A burden in several geographic settings. Since Brazil was the first Latin American country to introduce universal RV-A vaccination in March 2006 our data also highlight the need for maintaining the environmental surveillance and molecular characterization in order to follow the epidemiological pattern of RV-A excretion in the post-vaccination era.

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4.3 One year monitoring of norovirus in a sewage treatment plant in Rio de Janeiro, Brazil

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One year monitoring of norovirus in a sewage treatment plant in Rio de Janeiro, Brazil

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Objetivos:

- Avaliar a disseminação de NoV em águas residuárias da ETE-Fiocruz (Rio de Janeiro) e determinar a prevalência dos genótipos circulantes.

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ABSTRACT

Norovirus (NoV) is one of the most important aetiological agents of acute gastroenteritis both in developed and developing countries. NoV is shed in high concentrations by infected persons and contaminates recreational and drinking water through sewage discharge into the environment. The aim of this study was to determine the prevalence, genotypes and removal ratio of NoV by PCR, seminested-PCR and quantitative PCR (qPCR) assays in a sewage treatment plant in Rio de Janeiro city, Brazil, during one year of surveillance. NoV was detected in 7 (15%), 14 (29%) and 28 (58%) samples using PCR, seminested-PCR and qPCR, respectively. The mean removal ratio for the activated sludge process was 0.6 $\log_{10}$ for NoV genogroup I (GI) and 0.32 $\log_{10}$ for NoV genogroup II (GII). The peak NoV concentration was detected in the coldest months, with 53,300 genomic copies/litre. Nucleotide sequencing and phylogenetic analysis revealed that five strains clustered with GI strains and six with GII strains. This study demonstrates that NoV spreads into the environment despite the sewage treatment process and remains a source of waterborne outbreaks of acute gastroenteritis.

Key words | norovirus, quantitative PCR, sewage, treatment efficiency

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INTRODUCTION

Noroviruses (NoV) are the leading cause of nonbacterial outbreaks of acute gastroenteritis worldwide; they infect children and adults in both developed and developing countries. NoV is transmitted through the faecal-oral route by the consumption of contaminated water or food (Green *et al.* 2001). The virus has a non-enveloped capsid and possesses a positive single strand RNA genome of 7.7 kb with three open reading frames (ORF). ORF1 codifies a polyprotein that, after cleavage, gives rise to RNA-dependent RNA polymerase and viral protease. ORF2 encodes the major capsid protein, and ORF3 a minor capsid protein (Hardy 2005). NoV is classified in the Caliciviridae family, belonging to the *Norovirus* genus. Five genetically diverse genogroups (GI–V) have been described, with GI, GII and GIV infecting humans with 8, 17 and 1 genotypes, respectively (Zheng *et al.* 2006). In surveillance studies of acute gastroenteritis caused by NoV around the world, GII has been described as the most prevalent genogroup; however, the GI distribution remains largely unknown.

NoV is shed from patients with a median peak of 9.5×10^{10} genomic copies/g faeces in sewage (Atmar *et al.* 2008). Previous studies have determined the removal efficiency of the treatment process performed in sewage treatment plants (STP) for enteric viruses, and only showed small titre reductions when comparing influent vs. effluent samples (Haramoto *et al.* 2008; He *et al.* 2008). Owing to the lack of removal efficiency, enteric viruses can contaminate different types of environmental water, such as rivers and

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underground waters, resulting in an important risk for waterborne outbreaks of acute gastroenteritis.

The microbiological quality of different types of water is defined by the determination of total and faecal coliform levels present; however there is a scientific consensus that there is no correlation between bacterial indicators and viral contamination (Pusch *et al.* 2005; Carducci *et al.* 2008). This highlights the need for creating new regulations to determine which parameters must be accessed in order to certify good virological quality in water samples.

The detection of NoV is performed by molecular methods such as qualitative or quantitative PCR (qPCR) because of the lack of an efficient *in vitro* cell culture assay. Recently, a new approach was developed to culture viruses; however this methodology is in the early stage of development and needs to be tested with a broad range of NoV strains (Straub *et al.* 2007). The first qPCR for NoV quantification was described by Kageyama *et al.* (2003). The sensitivity of this assay permits the detection of up to 10 genomic copies/reaction, and is more sensitive than qualitative PCR. Several studies in environmental virology have used this technique, and the high sensitivity allowed for the detection of NoV at the low titres frequently found in those samples (Haramoto *et al.* 2006; Katayama *et al.* 2008).

The aim of this study was to determine the prevalence and circulating genotypes of NoV present in a STP in Rio de Janeiro city, Brazil, from January to December 2005, as well as the removal efficiency for that plant of enteric viruses, with NoV as a model.

MATERIALS AND METHODS

Sewage samples

From January to December 2005, 24 influent and 24 effluent composite samples were collected twice a month from a STP located in Rio de Janeiro city, Brazil. The treatment includes secondary aerobic digestion by activated sludge, extended aeration with biological nitrification and denitrification, without chlorination.

Each sample consisted of eight 250 ml portions that were stored in glass bottles and transported to the laboratory, where they were immediately tested for bacterial parameters. Within 24 hours, the samples were processed for viral concentration and stored at −80°C until RNA extraction for viral detection.

Bacterial parameters

Total and faecal coliforms were measured using the Colilert®-18 Quanti-Tray/2000 method (IDEXX Laboratories, Westbrook, Maine) according to the manufacturer's instructions.

Viral concentration, RNA extraction and cDNA synthesis

The viruses were concentrated using an adsorption-elution method with a negatively charged membrane as described previously by Katayama *et al.* (2002). After concentration, the eluate (15 ml) was ultrafiltered using a Centriprep concentrator 50® (Nihon Millipore, Tokyo, Japan) to a final volume of 2 ml.

The samples were clarified by adding 70 µl of Vertrel® (Sigma-Aldrich Corporation, St Louis, Missouri) and centrifuged at 800 × g for 10 min. RNA was extracted by using the QIAamp Viral RNA Mini Kit® (QIAGEN, Valencia, California) following the manufacturer's instructions. The cDNA synthesis was performed with random primers pd(N)6 (Amersham Biosciences, Piscataway, New Jersey) at 42°C for one hour and 95°C for 10 minutes as previously described by Victoria *et al.* (2009).

Recovery efficiency of the method for the concentration of norovirus

To determine the recovery efficiency for NoV of this adsorption-elution method followed by the ultrafiltration procedure, 100 µl of a 10% faecal suspension of a GII/4 NoV strain (GenBank accession number DQ997040) previously quantified by qPCR was spiked in raw and treated sewage samples. Negative control without spiking NoV was also tested in order to certify the absence of a natural contamination. All the assays for viral recovery efficiency were carried out in triplicate (independent experiments) for each type of water (raw or treated samples).

PCR, seminested-PCR and qPCR reactions

For NoV detection, three different protocols were carried out. A PCR was performed by using a set of degenerate primers (Mon 431/Mon434) previously used to detect the most common strains of NoV from GI and GII according to the following conditions: denaturation for 3 minutes at 94°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 50°C, one minute at 72°C and a final extension for 10 minutes at 72°C (Beuret *et al.* 2002). A second detection method performed in this study was based on a seminested-PCR and used to discriminate between GI and GII (Boxman *et al.* 2006). The fist round of the seminested-PCR was carried out by denaturing the cDNA at 94°C for 5 minutes followed by 40 cycles at 94°C for 30 seconds, 45°C for 30 seconds and 72°C for one minute; the final elongation was at 72°C for 10 minutes. The second round was performed with 35 cycles with the same conditions of the first round of the seminested-PCR. These two PCR protocols use primers targeting the RNA polymerase region on the NoV genome.

The qPCR reactions were performed in duplicate using the ABI 7500 Real-Time PCR System® (Applied Biosystems, Foster City, California) following the manufacturer's instructions. The assay performed for NoV quantification was carried out by using the protocol described by Kageyama *et al.* (2003) that uses primers targeting the ORF1/ORF2 overlap region in the viral genome. The reaction was performed at 50°C for two minutes to activate UNG, followed by an initial denaturation at 95°C for 10 minutes, 45 cycles with denaturation at 95°C for 15 seconds and annealing/extension at 56°C for one minute.

For all amplification procedures, basic precautions were taken to avoid cross-contamination among samples; these included the use of separated rooms for each PCR step and the inclusion of negative (milli-Q water) and positive controls (specific NoV GII and GI positive faecal samples previously tested) in all procedures.

Sequencing and phylogenetic analysis

The amplicons obtained from the seminested-PCR reactions were sequenced using Big Dye® Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, California) and ABI Prism 3730 Genetic Analyzer® (Applied Biosystems) as described by Otto *et al.* (2008). BioEdit® Sequence Alignment Editor software was used to edit and align the sequences obtained in this study (Hall 1999). Phylogenetic analyses were carried out by using the MEGA v. 4.0 software package (Tamura *et al.* 2007) with the neighbor-joining method, Kimura 2-parameters model for distance correction and 2,000 bootstrap re-sampling test. Sequences from GI (187 nt) and GII (237 nt) obtained in this study were submitted to GenBank under accession numbers FJ429186 to FJ429197.

RESULTS

The prevalence and genotypes of NoV were determined in a one year monitoring study in a STP in Rio de Janeiro city, Brazil. A total of 48 samples were collected: 24 influent and 24 effluent. The recovery efficiency for NoV of the adsorption-elution method followed by the ultrafiltration procedure used in this study was 7.8% and 6.4% for raw and treated sewage samples, respectively (data not shown). Comparing PCR, seminested-PCR and qPCR, the most sensitive method for detection of NoV in these sewage samples was qPCR, which detected a total of 28 (58%) positive samples for NoV; of these, 16 were (67%) positive for GII and 1 (4%) for GI in influent samples; and 10 (42%) for GII and 1 (4%) for GI in effluent samples. When seminested-PCR was performed, NoV was detected in 14 (29%) samples, with 8 (33%) positive for GII and 2 (8%) for GI in influent samples and 3 (12%) for GII and 3 (12%) for GI in effluent samples. Two samples were concomitantly positive for both GI and GII genotypes. The PCR detected 6 (25%) samples positive for NoV in influent samples and 1 (4%) in effluent samples (Table 1). For qPCR, the mean titres of NoV GII in influent and effluent samples were 7,290 genomic copies/l ($gc\,l^{-1}$) and 3,470 $gc\,l^{-1}$, respectively. For NoV GI, the values observed were 2,400 $gc\,l^{-1}$ and 643 $gc\,l^{-1}$, respectively. The mean removal ratio for the treatment process was 0.6 $\log_{10}$ for NoV GI and 0.3 $\log_{10}$ for NoV GII. For total and faecal coliforms, the mean removal ratios were 2.5 $\log_{10}$ and 3.2 $\log_{10}$, respectively.

When using qPCR, NoV GII was detected in all influent samples except that collected in January. A peak in concentration was observed during the coldest/driest

Table 1 | Norovirus positive samples detected by PCR, seminested and quantitative PCR (qPCR) in influent and effluent samples from the sewage treatment plant in Rio de Janeiro, Brazil

	Norovirus detection using each methodology				
		Seminested-PCR		QPCR	
Samples (n)	PCR	GI	GII	GI	GII
Influent (24)	6	2	8	1	16
Effluent (24)	1	3	3	1	10
Total (48)	7 (14.6)*	14 (29.2)*		28 (58.3)*	

*Total samples with percentage in parentheses.

months of the year (June and July). Comparatively, when effluent samples were analysed, NoV GII was not detected in a five month period (Figure 1).

All positive samples for NoV (11 for GII and 5 for GI) detected by seminested-PCR were sequenced and confirmed as NoV by comparison with sequences available in GenBank using the BLAST program. Phylogenetic analyses were carried out for molecular characterization of detected NoV genotypes. The GII strains detected in this study clustered in four different groups (Figure 2). The strains clustered with GII/4 prototype strains presented 100% nucleotide identity and 98% identity with the most similar sequence identified from GenBank; this strain was isolated in Canada in 2005 and belonged to the GII/4 genotype. The sequences from samples 70 and 71 were identical, confirming the presence of the same NoV strain in influent and the effluent samples from the same collection date; together

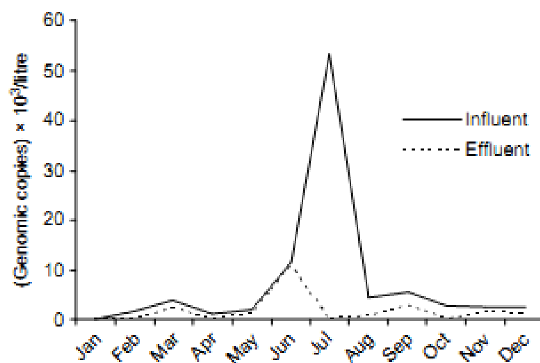

Figure 1 | Average concentration of norovirus genogroup II in influent and effluent samples from January to December 2005 at the sewage treatment plant in Rio de Janeiro, Brazil.

with the sequence from sample 107, these viruses formed a cluster without any reference strain. Strains 70 and 71 were more similar to a strain isolated in Stockholm in 2002 that clustered with GGIIb variants. Strain 105 clustered with a GII/3 reference strain, presenting the highest nucleotide identity (97%) with a strain isolated in Mexico in 2006 from a sea water sample.

The sequences belonging to GI clustered with two different sets of strains. Samples 56 and 57 were detected on the same collection date in both influent and effluent samples; they presented 99% nucleotide identity. These samples were more similar to a strain isolated in Japan, with 97% of identity in the nucleotide sequence, and clustered with the Southampton (GI/2) reference strain. The sewage strains 103, 104 and 110 were identical and presented 95% nucleotide identity with a strain isolated in the USA. They did not cluster with any reference strain (Figure 3).

DISCUSSION

To our knowledge this is the first study performed in Brazil to detect NoV in sewage samples collected from a STP. The prevalence, genotypes, seasonality and removal ratio of NoV were determined. The use of an adsorption-elution method for concentration of enteric viruses from environmental water samples followed by viral detection with molecular methods such as qualitative and quantitative PCR have been previously applied successfully (Haramoto *et al.* 2006; Silva *et al.* 2007; Villar *et al.* 2007; Miagostovich *et al.* 2008). Katayama *et al.* (2002, 2008) described the adsorption-elution method and obtained satisfactory results for detection of NoV in six STPs with an activated sludge process in Japan. The NoV recovery efficiency of 7.8% and 4.6% for raw and treated sewage samples, respectively, was similar to the astrovirus recovery of 4.2% and 4.3% for untreated and treated sewage samples, respectively, obtained in a previous study (Guimarães *et al.* 2008). Three different approaches for NoV detection were compared for sewage samples. The qPCR using TaqMan® technology presented a higher sensitivity when compared with both PCR and seminested-PCR; however none of these assays determined the infective capacity of the viral particles. The primary advantage of using real time PCR is

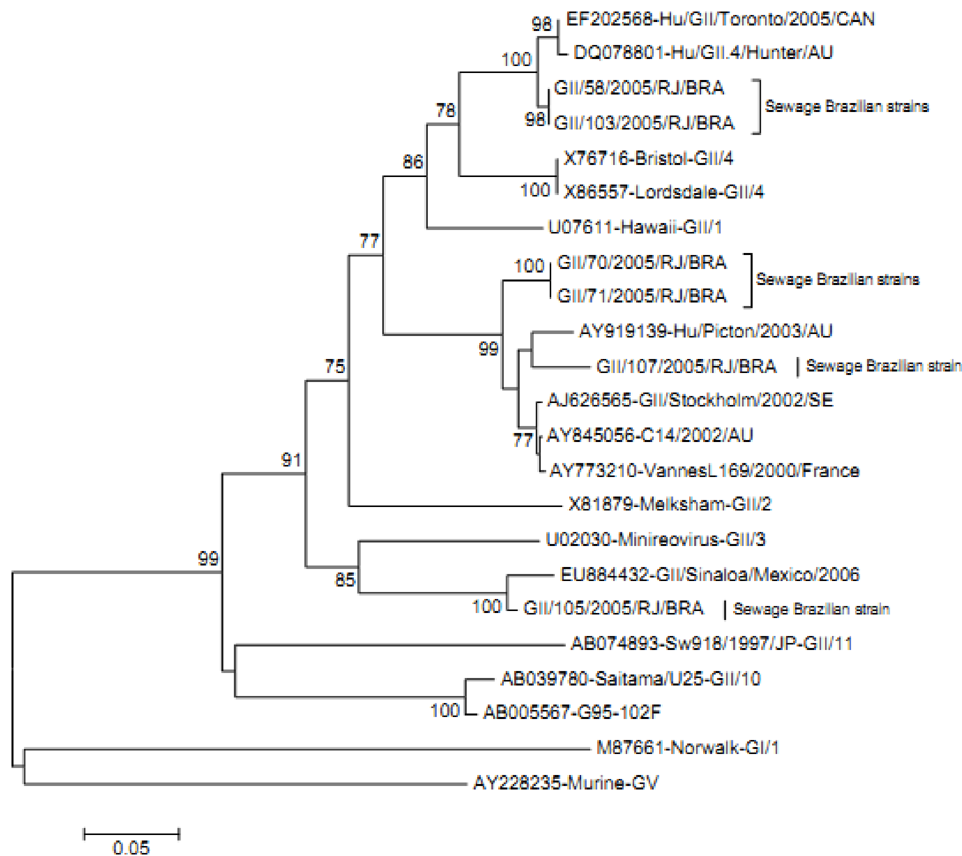

Figure 2 | Phylogenetic tree of 237 nucleotides within the polymerase region of norovirus genogroup II (GII) strains collected from a sewage treatment plant in Rio de Janeiro in 2005 and strains obtained from GenBank. Reference strains are indicated with the Genbank accession number followed by genogroup/genotype (in bold). Bootstrap values higher than 70% are given for each node.

that it permits the quantification of viral particles in the sample; this is currently the only way to achieve NoV quantification, since there is no efficient *in vitro* cell culture technique for these viruses (Straub *et al.* 2007). The qPCR protocol used in this study was described by Kageyama *et al.* (2003) for detection of NoV in stool specimens, and similarly to the present study, they observed a higher sensitivity (10 genomic copies/qPCR reaction) when compared with the nested PCR used in the study. The data obtained here highlight the importance of selecting the correct molecular detection method for NoV.

A seasonal profile was observed for the quantification of NoV GII from influent samples, with the peak of virus load in July. This was in accord with previous studies suggesting a higher prevalence of NoV in winter months in environmental samples (Haramoto *et al.* 2006; Silva *et al.* 2007; Katayama *et al.* 2008). Sewage samples were positive for NoV in all periods analysed. However, during the surveillance of NoV in stool samples from patients in three public hospitals in the city of Rio de Janeiro carried out by our laboratory in 2005, NoV was not detected in January and November (data not shown). The absence of clinical cases can be explained by NoV excretion by asymptomatic individuals with resulting contamination of the environment as previously described by Okabayashi *et al.* (2008), or by the location of the STP and the hospitals in different regions of the city. Effluents contaminated with NoV can spread to recreational and drinking waters, providing a

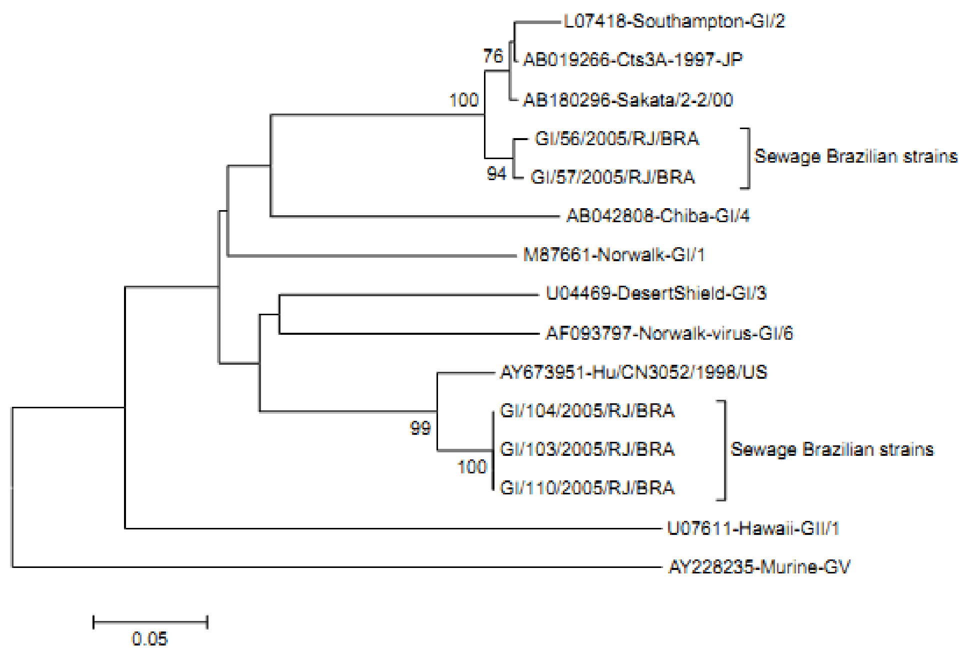

Figure 3 | Phylogenetic tree of 187 nucleotides within the polymerase region of norovirus genogroup I (GI) strains collected from a sewage treatment plant in Rio de Janeiro in 2005 and strains obtained from GenBank. Reference strains are indicated with Genbank accession number followed by genogroup/genotype (in bold). Bootstrap values higher than 70% are given for each node.

source for acute gastroenteritis outbreaks in the vicinity of the STP.

The removal of different types of microorganism was determined for the STP analysed in this study; this STP uses a primary sedimentation and biological treatment with activated sludge. The removal ratio for bacterial indicators was nearly six times higher than for NoV removal; these findings are in agreement with previous studies that indicate a low removal of enteric viruses from STPs (Berg *et al.* 2005; Haramoto *et al.* 2008). NoV GII was detected year-round and was more prevalent than GI, as previously described in both clinical and environmental trials worldwide (Berg *et al.* 2005; Lodder & de Roda Husman 2005; Victoria *et al.* 2007; Patel *et al.* 2008). Chan *et al.* (2006) observed that the viral load of NoV GII is 100-fold higher than that of GI strains in stool specimens of patients with acute gastroenteritis, and suggested that this higher GII viral load facilitates the transmission from infected to susceptible persons. It is likely that this higher transmission rate is responsible for the higher prevalence of GII worldwide.

Shin & Sobsey (2008) suggested that a water chlorination process could improve the inactivation of most enteric viruses, including NoV, present in sewage following the recommendation of the United States Environmental Protection Agency (USEPA Guidance Manual 1989).

Several strains from GI and GII were detected in both influent and effluent samples collected from the STP, indicating co-circulation of these strains in the population of Rio de Janeiro city. A previous study performed in this city using stool specimens from hospitalized children with acute gastroenteritis in 2004 indicated that numerous strains circulated in this set of patients (Victoria *et al.* 2007). Similar results were observed in a study conducted in the Netherlands, which detected 11 different NoV genotypes in sewage samples and up to four different NoV genotypes in a single sample (Berg *et al.* 2005). In the present study, the same NoV strain sequences were detected twice in influent and effluent samples collected on the same date, confirming the resistance of the virus to the sewage treatment process. The seminested-PCR assay permitted the

detection of GII and GI strains in the same sample; this detection was confirmed by sequencing.

The NoV discharged in STP effluents can reach recreational or oyster culture waters. Since recombination is a frequent mechanism of evolution in NoV biology (Bull *et al.* 2007; Nayak *et al.* 2008), the consumption of these waters or oysters containing two strains belonging to different genogroups could be a source of recombination events that could lead to worldwide outbreaks of acute gastroenteritis.

CONCLUSIONS

This study demonstrates the presence of different genotypes of NoV in sewage samples collected in a STP, and highlights that viral contamination can persist after sewage treatment performed with primary sedimentation followed by biological treatment. It is therefore important to consider the risk for local population health concerning outbreaks of waterborne acute gastroenteritis.

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4.4 Molecular detection, quantification and characterization of human polyomavirus JC from waste water in Rio de Janeiro, Brazil

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Molecular detection, quantification and characterization of human polyomavirus JC from waste water in Rio De Janeiro, Brazil

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Objetivos:

- Estabelecer metodologias de detecção, quantificação e caracterização molecular de JCPyV e avaliar a disseminação destes vírus em águas residuárias da ETE-Fiocruz (Rio de Janeiro).

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ABSTRACT

Polyomavirus JC (JCPyV) is largely excreted by the human population through the urinary route and has been recognized as a potential viral marker for human waste contamination. This study aims to investigate the dissemination of JCPyV in waste water from a sewage treatment plant (STP) located in Rio de Janeiro, Brazil, and to describe the prevalence of JCPyV subtypes currently present in this population. Raw and treated sewage samples were collected bimonthly during one year, and examined for the presence of JCPyV using nested polymerase chain reaction (nPCR) and quantitative real time PCR (qPCR). JCPyV was detected by nPCR in 96% and 43% of raw and treated sewage samples, respectively. The concentration of JCPyV present in the samples ranged from 1.2×10^3 to 3.2×10^5 and 2.6×10^2 to 6.2×10^3 genome copies per 2 ml of concentrated raw and treated sewage sample, respectively. The strains were characterized and the obtained nucleotide sequences indicated that the detected JCPyV strains clustered with subtypes of East African, West African and European origin. To our knowledge, this is the first study describing the incidence and diversity of JCPyV strains in raw and treated sewage in Brazil.

Key words | Brazil, JCPyV, polyomavirus, quantitative PCR, waste water

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INTRODUCTION

Polyomavirus JC (JCPyV) belongs to the family *Polyomaviridae*, which is classified as a group 1 virus in the Baltimore classification scheme. It is non-enveloped with a genome that encompasses a single 5.1 kb molecule of circular double-stranded DNA (Neu *et al.* 2009). *Polyomavirus* is a single genus designation in this virus family and this genus contains 14 different species that infect at least 8 different mammalian species.

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Recently, three new human polyomaviruses (WUPyV, KIPyV and MCPyV) have been described (Allander *et al.* 2007; Gaynor *et al.* 2007; Feng *et al.* 2008). JCPyV were associated initially with progressive multifocal leukoencephalopathy (PML) and the role of these viruses in the development of human cancer has been suggested (Imperiale 2000). Seroepidemiological studies have shown that JCPyV infection is widespread in the human population, with a

prevalence rate ranging from 58 to 92% in adults (Shah *et al.* 1997; Knowles 2006; Egli *et al.* 2009).

The transmission of human polyomaviruses is still unknown. It has been reported that JCPyV can replicate in tonsillar B lymphocytes and stromal cells, supporting the notion that the respiratory tract is the primary site of viral infection (Monaco *et al.* 1998). A study performed in urban sewage from different geographical areas detected the high level of JCPyV excretion and supports the idea previously described that urine–oral transmission will probably happen soon in vivo, inside the family or from closely related people and less frequently later in life from other polluted sources (Bofill-Mas *et al.* 2000). Zheng *et al.* (2004) supported that the transmission of JCPyV is via a parent-to-child transmission during cohabitation.

Primary infection occurs during childhood and is mostly subclinical. Following infection, the virus persists asymptomatically in renal tissue (Arthur & Shah 1989; Kitamura *et al.* 1990). Reactivation of JCPyV can occur at any time in life, as demonstrated by the detection of viral progeny in the urine of a high percentage (20–80%) of healthy individuals over 30 years of age (Agostini *et al.* 2001; Pavesi 2005).

Human JCPyV infections appear to be population-associated in that the genotype of JCPyV excreted by individuals of defined ethnicities is in high proportion determined by the geographical origin of the ethnic group rather than the JCPyV genotypes that are prevalent in their current location (Bofill-Mas *et al.* 2000).

This study determines the prevalence of JCPyV in waste water from a sewage treatment plant (STP) in order to elucidate the circulation of these viruses in the local population. Waste water is the main source of pathogenic microorganisms, and thus provides information about the different strains that are infecting a population. For this purpose a one-year study was carried out in an urban STP in the city of Rio de Janeiro. Urine samples from the local population collected during the same year were analysed to compare the genotypes of JCPyV from these human and environmental samples.

To our knowledge this is the first study demonstrating the circulation of JCPyV in sewage samples in South America.

MATERIALS AND METHODS

Sewage samples

From January to December 2005, a total of 47 sewage composite samples, 24 from raw and 23 from treated sewage, were collected twice a month from an activated sludge STP in the city of Rio de Janeiro, Brazil, as described by Guimaraes *et al.* (2008). One sample from treated sewage was not in sufficient quantity to test for JCPyV. The treatment of the STP studied includes secondary aerobic digestion by activated sludge, and extended aeration with biological nitrification and denitrification, without chlorination.

Clinical samples

JCPyV detected in 12 urine clinical samples that previously tested positive using a nested PCR to amplify the T antigen region of the DNA genome (Weber *et al.* 1994) were genetically characterized. All clinical samples were obtained from HIV-1 and/or HTLV-1 patients from an Infectious Disease Public Hospital located in the same area of the STP. All patients were Brazilian citizens with ages ranging from 29 to 63 years old and four of them presented symptoms of PML disease.

Virus particle concentration method

Viral particles present in waste water samples (affluent and effluent) were concentrated using an adsorption-elution method with negatively charged membranes, which included the insertion of an acid rinse step for removal of cations, as described previously (Katayama *et al.* 2002). Briefly, $MgCl_2$ was added to every sample to a final concentration of 3 mM, passed through a fibreglass membrane AP20 (Nihon Millipore®, Tokyo, Japan) to eliminate large-particulate, then through an HA (mixed cellulose esters) negatively charged membrane filter (0.45 mm pore size and 142 mm diameter Nihon Millipore®). The filter was rinsed with 350 ml of H_2SO_4 (0.5 mM, pH 3.0) and the viruses were eluted with 15 ml of NaOH (1 mM, pH 10.8). The filtrate was recovered in a tube containing 50 µl of H_2SO_4 (100 mM, pH 1.0) and 50 µl of 100 × Tris–EDTA buffer (pH 8.0) for pH neutralization, followed by

centrifugation using a Centriprep YM-50® (Millipore) at 1,500 g for 10 min to obtain a final concentrate volume of 2 ml.

Extraction of viral DNA

The viral genome DNA was extracted from pre-treated sewage samples using Vertrel® (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) with a QIAmp Viral DNA Mini Kit® (Qiagen, Inc., Valencia, California, USA) following the manufacturer's protocol as previously described (Guimaraes *et al.* 2008).

Real-time PCR standard curve and viral titration

A pBR322 plasmid containing the whole genome of JCPyV (Mad-1 strain) was used to construct the standard curve (SC) in the quantitative assay (Albinana-Gimenez *et al.* 2006). To prepare the human JCPyV DNA SC, *E. coli* Top 10 cells were transformed with the plasmid pBR322 containing the full JCPyV genome. The plasmids were purified from the bacteria using the commercial Kit Maxiprep Purification System® (Promega Corporation, Madison, Wisconsin, USA) following the manufacturer's instructions and the DNA obtained was quantified with a NanoDrop® (Thermo Scientific) by absorbance at 260 nm. The copy number was estimated based on the plasmid and insert size (Yin *et al.* 2001). A JCPyV-specific SC was generated by a 10-fold serial dilution (5×10^6 to 5×10^0 copies per reaction) of purified JCPyV (strain Mad-1) genome DNA. Final values for absolute levels of viral genome are given as genome copies (GC)/reaction.

Real-time PCR to detect human JCPyV DNA was performed as described previously (Pal *et al.* 2006). The protocol was adapted to the ABI 7500 (Applied Biosystems®, Foster City, California, USA) and performed using 5 µl of the DNA sample or 5 µl of the quantified plasmid DNA, 12.5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems®), and 500 and 150 nM concentrations of each primer and probe, respectively, in a final mixture reaction volume of 25 µl.

For all molecular procedures four separate rooms were used to avoid cross contamination of samples. The samples were analysed in duplicate, and the specific prototype Mad-1 DNA plasmid previously tested and milli-Q water were used as positive and negative controls, respectively, in all procedures.

Nested polymerase chain reaction (nPCR)

Qualitative nPCR was used for molecular detection and characterization of the JCPyV strain. The nPCR amplification was performed using a set of primers that target an intergenic region fragment spanning nucleotides (nt) 2099 to 2766 of the JCPyV genome and thermo cycling conditions, as previously described by Bofill-Mas *et al.* (2000). Every DNA extraction was diluted 10-fold, and then 5 µl of this dilution was analysed for the presence of inhibitors in sewage samples.

JCPyV sequence reaction and phylogenetic analysis

The amplicons (668 bp) obtained in the nPCR were purified using the QIAquick® PCR Purification Kit (Qiagen, Inc.) following the manufacturer's recommendations and quantified with a NanoDrop® (Thermo Scientific) by absorbance at 260 nm. PCR products were sequenced using an ABI Prism® 3100 Genetic Analyzer and Big Dye® Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems) in both directions using individually the same primers as in the amplification reactions. Centri-Sep Columns (Princeton Separations, CA, USA) were used for purification of the sequencing reaction products, according to the manufacturer's recommendations.

The nucleotide (nt) sequences determined in this study were aligned and edited using the BioEdit Sequence Alignment Editor, deposited in the GenBank database and compared with available sequences including reference sequences of each genotype. A phylogenetic tree was constructed using the MEGA program version 3.1 by the neighbour-joining method, with the genetic distance calculated by the Kimura 2-parameter model using bootstraps analysis with 2000 pseudo-replicas (Kumar *et al.* 2004).

Nucleotide sequences obtained in this study from sewage (30/32) and clinical samples (8/12) were submitted to National Center for Biotechnology Information (GenBank, http://www.ncbi.nlm.nih.gov/) under the accession numbers FJ524336 to FJ524365 and FJ524366 to FJ524373, respectively.

Table 1 | Detection and quantification of polyomavirus JC (JCPyV) in sewage samples using nested polymerase chain reaction (nPCR) and quantitative PCR (qPCR)

	JCPyV detection		JCPyV quantification	
Sewage samples	nPCR positive/tested (%)	qPCR positive/tested (%)	Viral load	Media
Raw	23/24 (96)	23/24 (96)	1.2×10^3–3.2×10^5	4.6×10^4
Treated	10/23 (43)	9/23 (39)	2.6×10^2–6.2×10^3	2.4×10^3
Total	33/47 (70)	32/47 (68)	–	–

RESULTS

Detection and quantification of JCPyV

Table 1 shows the nPCR and qPCR results used to detect and quantify JCPyV from the 47 samples collected in the STP, 23 from affluent and 24 from effluent waste water. Using nPCR, viruses were detected in 96% (23/24) and 43% (10/23) from affluent and effluent waste water samples, respectively. The medium viral load in raw waste water samples was one logarithm higher than in the finished treated water, as expected in an STP. Table 2 shows the reduction of the concentration of JCPyV in the STP demonstrated for each month during the study. The values presented in log (10) represent the difference between the medium concentration of genome copies in raw and treated sewage per month. As observed in Table 2, no JCPyV could be detected during summer months in the treated samples.

The qPCR assay was also applied to the quantification of JCPyV in urine samples to calculate the viral load excretion from patients, and the data are expressed as GC per reaction. Concentration of JCPyV ranged from 1.26×10^1 to 5.19×10^6 GC/reaction. All 12 samples were confirmed as JCPyV positive using both nPCR and the quantitative assay.

Characterization of JCPyV

The amplicons of 668 bp obtained by nPCR (nt 2099 to 2766) were sequenced and confirmed as JCPyV by BLAST comparison with sequences available from GenBank using the BLAST program. JCPyV strains detected from sewage and clinical samples had a nucleotide identity with each other that ranged from 95.3 to 100%.

Phylogenetic analyses were carried out for molecular characterization of JCPyV genotypes. The results of the

Table 2 | Concentration of polyomavirus JC in the waste water treatment plant distributed along a one-year data collection

	Raw sewage		Treated sewage		
Month	GC per 2 ml	Std dev.	GC per 2 ml	Std dev.	Reduction*
January	5.66×10^3	2.42×10^3	ND†	–	3.75
February	4.71×10^3	3.63×10^2	ND†	–	3.67
March	2.97×10^3	8.79×10^2	ND†	–	3.47
April	7.21×10^3	7.66×10^3	ND†	–	3.85
May	3.98×10^3	–	5.14×10^2	–	0.89
June	1.71×10^4	1.93×10^4	2.57×10^2	–	1.82
July	1.19×10^5	–	6.00×10^2	–	2.30
August	2.95×10^4	2.48×10^4	4.37×10^3	2.54×10^3	0.83
September	3.83×10^4	3.91×10^4	4.19×10^3	1.22×10^2	0.96
October	1.69×10^4	2.22×10^4	1.45×10^3	1.20×10^2	1.06
November	2.75×10^5	6.99×10^4	ND†	–	5.44
December	4.18×10^4	3.24×10^4	ND†	–	4.62

*Accumulative elimination efficiency expressed in log (10).
†ND: Not detected.

maximum likelihood method applied to the phylogenetic analyses are show in Figure 1. The strains detected in this study clustered mainly in three different groups. Sequence analyses of sewage and clinical samples from the intergenic region of the JCPyV genome indicated that JCPyV clustered with subtypes of East Africa, West Africa and European origins.

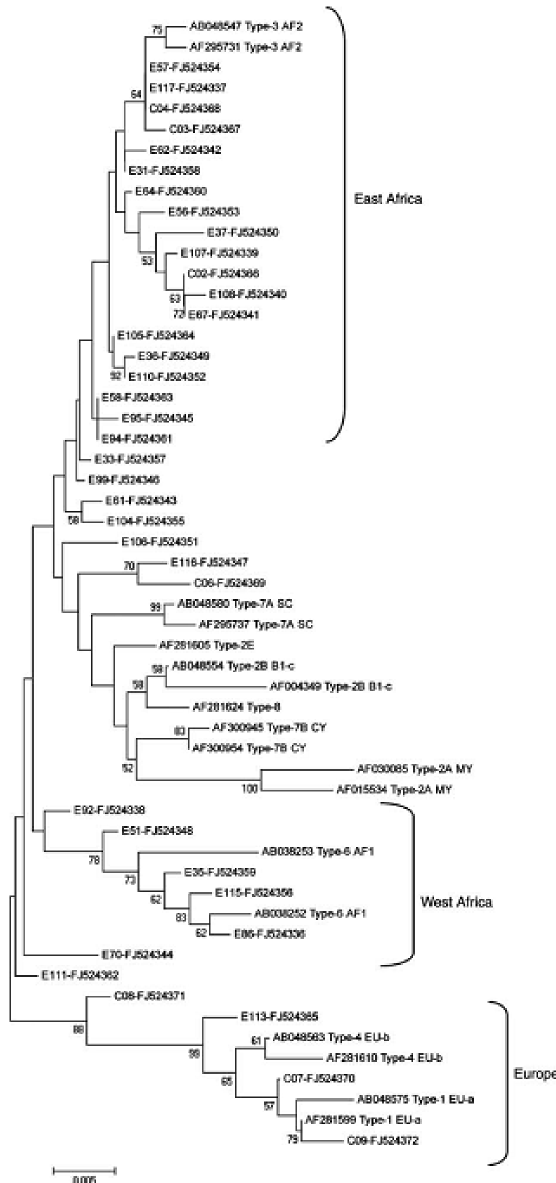

Figure 1 | Phylogenetic analyses of the intergenic regions of 38 sequenced JCPyV strains compared with previously described JCPyV prototypes. E or C before accession number means environmental and clinical samples, respectively.

DISCUSSION

The results produced in this study show a wide distribution of JCPyV infections in the area studied. JCPyV DNA was found in almost 100% of sewage samples, corroborating with previous studies that have reported high concentrations of viruses, mainly DNA viruses (adenovirus and polyomavirus), in residual waters worldwide (Bofill-Mas *et al.* 2000; Albinana-Gimenez *et al.* 2006; Bofill-Mas *et al.* 2006; Katayama *et al.* 2008; Rafique & Jiang 2008).

A correction factor was used to account for viral loss during the water concentration step. Based on this, virus levels found in sewage samples in this study presented a mean concentration of 10^6 and 10^4 GC/l for affluent and effluent samples, respectively. These virus concentrations were similar to concentrations found in samples of urban sewage in other geographical areas and corroborate with levels in the effluent of a waste water treatment plant (Bofill-Mas *et al.* 2006). The results clearly support the interest and applicability of using the specific human JCPyV as a microbial source tracking tool and as a marker of human faecal contamination as it has been suggested in previous studies, and furthermore for the evaluation of the virus removal efficiency in waste water treatment plants (Bofill-Mas *et al.* 2000).

The results also show that the methodologies (nPCR and qPCR) applied are feasible for the analysis of JCPyV as well as robust, allowing not only the quantification (96%) but also the genetic characterization of the identified samples. nPCR showed a slightly higher level of sensitivity than qPCR, and in treated sewage samples where concentrations of viruses are expected to be lower, more positive samples were detected by nPCR (70%) than by qPCR (68%). Previous data published elsewhere using the same samples showed a detection of 92% of hepatitis A virus (HAV) using qPCR, and 32% and 16% for treated and untreated samples, respectively (Villar *et al.* 2007). In another study, the percentage of norovirus detected ranged from 15% to 58%, depending on the methodology of detection used (Victoria *et al.* in press). Although we could not

demonstrate the recovery efficiency of the adsorption elution method for JCPyV, previous studies in this STP showed a recovery efficiency of 4.2% and 4.3% for raw and treated sewage samples for human astrovirus, and 7.8% and 4.6% for norovirus, respectively (Guimaraes *et al.* 2008; Victoria *et al.* in press). This same method, when used to concentrate waste water samples, presented satisfactory results for detection of enteric viruses in STPs in other studies (Haramoto *et al.* 2008; Katayama *et al.* 2008).

Treatments commonly applied in the STPs have significantly reduced the incidence of bacterial agents in finished water, but viral pathogens can persist following the treatment process, resulting in several human viruses being introduced back into the environment through the water system (Thompson *et al.* 2003; Bofill-Mas *et al.* 2006). Conventional waste water treatment plants that use filtration treatments and activated sludge have shown removal efficiencies of about two logarithms for JCPyV (Bofill-Mas *et al.* 2006). In this study the efficiency of the removal of viruses observed, in log (10), is highly variable ranging from 0.83 to 5.44. This variability could be explained by changes occurring in the amount of biological material observed in the STP during the summer time. In this biological treatment system, the rate of production of activated sludge is influenced by temperature: higher temperatures mean higher rates of growth. Thus, to maintain the same concentration of volatile suspended solids (measured by the amount of bacteria that degrade the sewage) there is a need for more disposal of sludge in the same period. Considering that the activated sludge adsorbs and enhances settling of material in suspension, we can explain the lower viral concentrations in the final effluent and the higher removal efficiency observed in those samples obtained from the warm season. Bacterial removal data obtained from these samples was previously published and showed to be 2.5 and 3.2 for total and faecal coliforms after sedimentation and biological secondary (activated sludge) treatment, respectively (Guimaraes *et al.* 2008).

The high prevalence of JCPyV in waste water, coupled with its high stability, qualifies this virus as a good marker for faecal pollution of human origin. The presence of JCPyV in the treated sewage shows that the virus can persist after the water treatment and that the treatment was unable to provide virus-free waste water effluent. The high stability of JCPyV in the environment, as described by Bofill-Mas *et al.* (2006) who demonstrated a t_{90} of 63.9 days and a t_{99} of 127.3 days for this virus in sewage samples, shows the importance in reducing or eliminating viral agents from treated effluent that will be discharged into other water sources. Information on the concentration of JCPyV in raw and treated waste water has been described as useful, not only in understanding their fate, but also in assessing the risk of infection through water (Maunula *et al.* 2005).

Monitoring the occurrence of JCPyV in sewage from a waste water treatment plant could be an appropriate advance to understanding the circulation of these viruses in the population located in the area enclosed by the STP, particularly because the influent contains viruses shed from patients of asymptomatic cases. It is considered that JCPyV has been evolving with the human population since the emergence of modern humans, 100,000–200,000 years ago. The major population groups in the Americas have shown, in previous studies that analysed urine samples, genotypes characteristic of their known Old World origins: European types 1 and 4, African types 3 and 6, and Asian type 2A (Stoner *et al.* 2000). However, a much wider search in South America was required to identify the distribution of genotypes in this geographical area. The analysis of the amplified sequences in the intergenic region of the JCPyV strains showed the presence of subtypes previously associated with populations of African and European origins, corroborating with the authentic structure of the Brazilian population and supporting the idea that the strains of JCPyV are related to the ethnic origins (Parra *et al.* 2003).

To our knowledge, this is the first study describing the incidence and diversity of JCPyV strains in raw and treated sewage in South America, and more specifically in Brazil. The information provided adds evidence to the applicability of JCPyV as a marker of human faecal pollution in widely divergent geographical areas.

CONCLUSIONS

Both molecular assays used in this study (nPCR and qPCR) support the applicability of either of these techniques to investigate the presence of JCPyV in the sorts of samples analysed in this study; quantification of JCPyV in untreated

sewage samples presented suitable results and corroborate previous findings that point to JCPyV as a possible viral marker to trace human faecal pollution in environmental scenarios; the high rates of viral reduction during the warm months suggest that operations in the STP, such as increasing the frequency of sludge removal, can help in the elimination of virus.

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4.5 Detection of rotavirus A in sewage samples using multiplex qPCR and an evaluation of the ultracentrifugation and adsorption-elution methods for virus concentration

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Protocol

Detection of rotavirus A in sewage samples using multiplex qPCR and an evaluation of the ultracentrifugation and adsorption-elution methods for virus concentration

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Objetivos:

- Estabelecer metodologia de quantificação (qPCR) para bacteriófago PP7 a fim de se avaliar sua utilização como controle interno nos métodos de recuperação viral.
- Estabelecer qPCR para RVA e avaliar esta técnica no formato multiplex para detecção simultânea de RVA e do controle interno PP7.
- Avaliar o método de ultracentrifugação para concentração de RVA comparando sua eficiência de recuperação com o método de filtração em membrana negativa.

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ABSTRACT

Group A rotaviruses (RV-A) are the most common agents of viral gastroenteritis in children worldwide. The goal of this study was to compare two different methods to concentrate RV-A from sewage samples and to improve the detection and quantification of RV-A using a multiplex quantitative PCR assay with an internal control. Both RV-A and the internal control virus, bacteriophage PP7, were seeded into wastewater and then concentrated using either an ultrafiltration-based adsorption-elution protocol or an ultracentrifugation-based protocol. Real time multiplex quantitative PCR was used to quantify the purified RV-A and PP7, and the results of the multiplex assay were compared with the results of the monoplex assays. The ultracentrifugation-based method had a mean recovery rate of 47% (range: 34–60%), while the ultrafiltration-based adsorption-elution method had a mean recovery rate of 3.5% (range: 1.5–5.5%). These results demonstrate that ultracentrifugation is a more appropriate method for recovering RV-A from wastewater. This method together with the multiplex qPCR assay may be suitable for routine laboratory use.

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1. Introduction

More than 140 virus types that cause a broad variety of illnesses in humans can be found in water as a result of contamination with waste (Gerba et al., 1996; Kukkula et al., 1997). Sewage influent contains pathogens shed by infected individuals, and the molecular detection and characterization of human viruses in urban sewage has been used extensively to derive information on the viruses circulating in a given population (Bofill-Mas et al., 2000; Ferreira et al., 2009; Fumian et al., 2010; Pintó et al., 2007; Rodríguez-Díaz et al., 2009; Victoria et al., 2010; Villena et al., 2003). Several of these viruses have been implicated in waterborne outbreaks of gastroenteritis including rotavirus A (RV-A) (Gerba et al., 1996; Hewitt et al., 2007; Kukkula et al., 1997; Villena et al., 2003). Despite the difficulty in determining the proportion of gastroenteritis cases resulting from waterborne infection, a significant percentage of the cases can be associated with poor water quality (Bosch et al., 2008). RV-A has been detected in environmental samples using different methods for virus concentration and detection (Ferreira et al., 2009; Kittigul et al., 2005; Mehnert et al., 1997; Villena et al., 2003).Natural inhibitors present in environmental samples and/or reagents used to concentrate viruses can be an obstacle for detecting viruses from these samples by interfering mainly in the molecular biology-based methods used for virus detection (Ijzerman et al., 1997; Schwab et al., 1995). A relatively low initial cost method based on adsorption-elution/ultrafiltration and elution with inorganic reagents has been shown to minimize this problem (Katayama et al., 2002). While this method favors the detection of viruses using molecular methods, it has the disadvantage of requiring two steps of virus concentration. This increases the cost per analysis, and purifying viruses from samples with a high amount material in suspension can be difficult. However, using this method, several authors have recovered viruses from different water matrices, including wastewater samples, at a low rate (Guimarães et al., 2008; Haramoto et al., 2006; Katayama et al., 2008; Miagostovich et al., 2008; Victoria et al., 2009, 2010; Villar et al., 2007).The main objective of this study was to compare the percentage of RV-A that could be recovered from

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wastewater using the either the adsorption-elution of the virus from negatively charged membranes or the ultracentrifugation-based concentration method that has been used largely to detect enteric viruses in wastewater (Bofill-Mas et al., 2006; Pina et al., 1998; Rodríguez-Díaz et al., 2009). The initial cost to obtain an ultracentrifuge and the ability to analyze only small sample volumes are two disadvantages of this method. However, the advantages of this method include the low cost per sample (all of the consumables used in this assay can be autoclaved and reused) and the lack of a second concentration step (Pina et al., 1998; Wyn-Jones and Sellwood, 2001).

The use of a bacteriophage PP7 as an internal control (IC) was also evaluated in both concentration methods. An IC is an important tool for molecular diagnostic assays to avoid the false negative results associated with enzymatic inhibition (Hoorfar et al., 2004). A negative IC result suggests failure during the concentration, extraction and/or PCR procedures. Additionally, a multiplex qPCR-based protocol was compared to using monoplex assays that have been used previously to detect RV-A and PP7 in order to further reduce the cost of the assay (Rajal et al., 2007; Zeng et al., 2008).

2. Materials and methods

2.1. Viruses and sewage samples

RV-A, G1P8 Wa prototype, was used for the construction of the standard curve.

For the spiking experiments a RV-A G1P8 isolate, isolated from an acute case of gastroenteritis using molecular techniques and confirmed by sequencing, was used (Genbank accession no. GU831596).

PP7 bacteriophage was provided kindly by Dr. Verónica Rajal (Salta University, Argentina), and a large amount was obtained by culture in the host *Pseudomonas aeruginosa* (ATCC 15692) using a protocol described previously (Rajal et al., 2007).

Raw sewage samples were collected from a sewage treatment plant in Rio de Janeiro, Brazil in order to perform the studies to determine the efficiency of the adsorption-elution and ultracentrifugation methods. Fourteen additional samples were collected at the same plant and stored in glass bottles at 4 °C until processed.

2.2. Virus concentration methods

RV-A and PP7 were concentrated using either an adsorption-elution/ultrafiltration method or an ultracentrifugation-based method. The adsorption-elution/ultrafiltration method was based on negatively charged membranes and included the addition of an acid rinse step for the removal of cations, as described previously by Katayama et al. (2002). The ultracentrifugation-based method was based on the protocol of Pina et al. (1998). In both cases, 42 mL of raw sewage was used as the starting material.

For the ultrafiltration-based method, the starting volume of sewage was diluted with 2 L of distilled water to facilitate the filtration process. Briefly, prior to filtration, $MgCl_2$ was added to a final concentration of 25 mM and the pH was adjusted to 5.0. The samples were filtered through a type HA negatively charged membrane (Nihon Millipore, Tokyo, Japan) with a 0.45 μm pore size using a vacuum pump system. The membrane was rinsed with 350 mL of 0.5 mM H_2SO_4 (pH 3.0), and 15 mL of 1 mM NaOH (pH 10.8) was used to release the viruses from the membrane. To neutralize the solution, 50 μL of 50 mM H_2SO_4 and TE buffer (100×; pH 8.0) was added. The eluate was filtered using a Centriprep Concentrator 50 (Nihon Millipore, Tokyo, Japan) and centrifuged at 1500 × *g* for 10 min at 4 °C to obtain a final volume of 2 mL. The filtration system was soaked briefly in a 10% bleach solution and rinsed with deionized H_2O prior each use.

For the ultracentrifugation-based method, 42 mL of sewage was ultracentrifuged at 100,000 × *g* for 1 h at 4 °C using a Beckman ultracentrifuge equipped with a type 35 rotor. Viral particles were resuspended in 3.5 mL of 0.25 N glycine buffer (pH 9.5) and incubated on ice for 30 min. The solution was neutralized by the addition of 3.5 mL of 2× phosphate-buffered saline (PBS, pH 7.2). The supernatant was clarified by centrifugation (12,000 × *g* for 15 min), and the viruses were finally recovered by ultracentrifugation at 100,000 × *g* for 1 h at 4 °C in an SW41 rotor. Viral particles were resuspended in 200 μL of 1 × PBS pH 7.2 and processed immediately for nucleic acid extraction or stored at −80 °C until use.

2.3. Spiking experiments

Two experiments performed in triplicate, on different days, were carried out to evaluate the recovery rate of both methods. One mL of a RV-A-containing fecal sample was spiked into the sewage samples, and one unseeded sample was used as negative control. The total amounts of both RV-A and PP7 were determined using both monoplex and multiplex qPCR. Part of the stool sample used to spike the sewage samples was used for RNA extraction and quantification together with the concentrated sewage samples. The number of viral particles was determined by adjusting the values according to the volumes used for each step of the procedure (extraction, cDNA synthesis and qPCR reaction). The estimated virus recoveries were calculated as the percentage of the number of copies of the inoculated RV-A recovered.

Natural contamination (i.e., pre-existing RV-A in the sewage sample) was quantified from the unseeded controls, and these values were subtracted from the total amounts to correct the results of the seeded samples.

2.4. Extraction of viral RNA and reverse transcription (RT)

Viral RNA was extracted from 200 μL of concentrated virus using the glass powder method (Boom et al., 1990). cDNA synthesis was carried out using reverse transcription with random primers (PdN6 – 50 A260 units – Amersham Biosciences, Chalfont St. Giles, Buckinghamshire, UK), as described previously (Ferreira et al., 2008). cDNA was prepared using RNA and a 1:10 dilution of RNA to investigate the presence of inhibitors in the sewage samples.

2.5. Real time multiplex quantitative PCR (qPCR)

The primers and probes used to detect RV-A and PP7 have been described previously by Zeng et al. (2008) and Rajal et al. (2007), respectively. The VIC-labeled RV-A probe, designed to target a highly conserved region of the non-structural protein 3 (NSP3), was synthesized by Applied Biosystems (CA, USA), and the PP7 primers were designed to amplify a region of the PP7 replicase gene (Table 1). The generation of the plasmids and the construction of the standard curve were performed as described previously by Fumian et al. (2009).

Multiplex qPCR was carried out using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, CA, USA). A standard curve (SC; 10^7, 10^5, 10^3 and 10^1 copies per reaction) was generated using tenfold serial dilutions of pCR2.1 vectors (Invitrogen, USA) containing either the RV-A NSP3 gene or the PP7 replicase gene. The optimal concentration of the primers and probe for RV-A was 400 nM and 200 nM, respectively; the optimal concentration of the primers and probe for PP7 was 500 nM and 120 nM, respectively. The multiplex qPCR reaction was performed in a 25 μL volume containing 12.5 μL of Universal PCR Master Mix (Applied Biosystems, CA, USA), 2.2 μL DNase/RNase-free water, 5 μL cDNA

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Table 1
Primers and probes used in the multiplex qPCR assay for the detection of RV-A and PP7.

Virus	Primer and probe	Sequence (5′ → 3′)	Size amplicon (bp)	Location
RV	NSP3 f	ACCATCTWCACRTRACCCTCTATGAG[a]		963–988
	NSP3 r	GGTCACATAACGCCCCTATAGC	86	1028–1049
	NSP3 probe	VIC-AGTTAAAAGCTAACACTGTCAAA-MGB		995–1017
PP7	247 f	GTTATGAACCAATGTGGCCGTTAT		247–270
	320 r	CGGGATGCCTCTGAAAAAAG	73	320–339
	274 probe	FAM-TCGGTGGTCAACGAGGAACTGGAAC-TAMRA		274–298

[a] IUB code: W = A/T, R = A/G.

and 1.25 µL each primer and 0.3 µL probe for PP7 or 1 µL each primer and 0.5 µL probe for RV-A. The initial concentration of all primers and probes used in the multiplex qPCR mix was 10 µM. After initial incubations at 50 °C for 2 min to activate the uracil-N-glycosylase and at 95 °C for 10 min for denaturation, two-step PCR amplification was performed with 40 cycles of 94 °C for 15 s and 56 °C for 1 min. Amplification data were collected and analyzed using Sequence Detection Software version 1.0 (Applied Biosystems, CA, USA). The sensitivity was evaluated using tenfold serial dilution of viral RNA and performing single real time RT-PCR versus multiplex real time RT-PCR for RV-A and PP7.

For all molecular procedures, four separate rooms were used to avoid cross-contamination of samples. Milli-Q water (Invitrogen, USA) was used as a negative control, in all procedures.

2.6. *Semi-nested RT-PCR*

Semi-nested RT-PCR for RV-A VP4 and VP7 detection has been used for molecular classification of RV-A into the G (VP7) and P (VP4) genotypes. The primers and protocols for this detection have been described previously (Das et al., 1994; Gentsch et al., 1992).

3. Results

3.1. *Establishment of the multiplex quantitative PCR assay*

In this study a multiplex qPCR assay was designed to detect RV-A and PP7 simultaneously. The sensitivity of the multiplex qPCR assay was evaluated in parallel experiments comparing the SC generated using purified DNA in a monoplex qPCR assay with the SC generated using the same cDNA in the multiplex qPCR assay and with results obtained from the sewage concentration tests. Table 2 lists the Ct values obtained for the standard curve using purified plasmid DNA to quantify RV-A and PP7 using the monoplex and multiplex strategies. No differences were observed over the range of DNA concentrations tested using these methods.

3.2. *Comparison of the concentration methods for rotavirus A recovery from residual water*

Using the ultracentrifugation-based method, natural contamination was observed in two unseeded samples used as negative controls (see Section 2). After concentration of the sample using this method, the qPCR assay detected a mean of 1.9×10^6 RV-A genomes per L of raw sewage and demonstrated a positive result until the tenfold dilution. In contrast, following concentration with the adsorption-elution method, naturally occurring RV-A was detected in just one sample, and the number of RV-A copies recovered was ten times less (1.8×10^5 cDNA copies per L of raw sewage) compared with the ultracentrifugation-based method. These values for the pre-existing RV-A level in the samples were used to correct the total copy number determined following either concentration strategy (see Section 2).

In this way, the corrected recovery rate following the ultracentrifugation method was 47% (range: 34–60%), while the corrected recovery rate following the adsorption-elution method was 3.5% (range: 1.5–5.5%; Fig. 1).

3.3. *Natural occurrence of Rotavirus A*

All fourteen raw sewage samples concentrated using the ultracentrifugation method and tested by qPCR tested positive for RV-A and PP7. The number of RV-A genomes per L of raw sewage ranged from 2.5×10^4 to 1.6×10^7. The VP4 and VP7 genes were genotyped in all positive samples, and 14 samples were G2, five samples were P4 and five samples were P6. In four samples, the P genotype could not be determined.

4. Discussion

In the present study, the efficiency of two methods used to concentrate viruses from raw sewage samples spiked with RV-A and PP7 was evaluated. The multiplex qPCR assay detected as few as 30 RV-A and 10 PP7 genomes per reaction with Ct values of 34.82 and 37.51, which is comparable to the monoplex qPCR used in this study and the original published protocol (Rajal et al., 2007; Zeng et al., 2008).

The advantages of using the multiplex protocol for the simultaneous amplification and quantification of the target and IC sequences include reduced procedure time and reagent cost. Environmental sewage samples can potentially contain a large quantity of PCR inhibitors, which could cause false negative results (Ijzerman et al., 1997). Therefore, the IC may assist in the detection of inhibitors. The successful amplification of the IC confirms that a negative result obtained for the tested virus is not a false negative (Rajal et al., 2007; Rolfe et al., 2007). The usefulness of an IC to avoid false negative results has been recognized, and the development

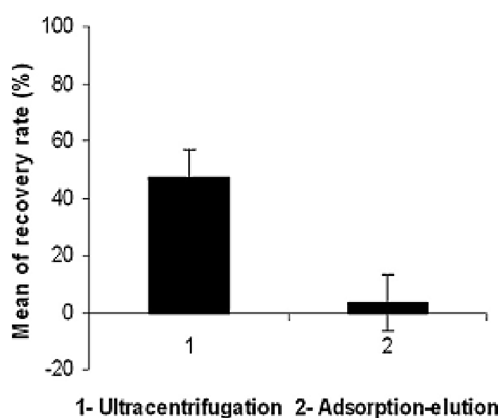

Fig. 1. The mean recovery rate of RV-A from sewage water following concentration using either the ultracentrifugation or adsorption-elution method ($n = 6$).

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Table 2
The mean Ct value obtained from the standard curve using either a monoplex or multiplex assay to detect rotavirus A (RV-A) and bacteriophage PP7 ($n = 6$).

Virus	PCR type	Quantity[a]/Ct value (sd[b])			
		10^7	10^5	10^3	10^1
RV-A	Monoplex	14.86 (0.030)	21.34 (0.10)	27.95 (0.121)	34.69 (0.55)
	Multiplex	15.15 (0.085)	21.87 (0.036)	28.50 (0.135)	34.82 (0.238)
PP7	Monoplex	16.62 (0.079)	23.13 (0.094)	29.49 (0.022)	36.28 (0.789)
	Multiplex	17.41 (0.086)	23.01 (0.030)	30.52 (0.079)	37.51 (0.392)

[a] Presented as the genome copy number per reaction.
[b] Standard deviation.

of a multiplex qPCR assay to detect RV-A and an IC simultaneously would meet the conditions for a suitable method for routine laboratory use: sensitivity, reproducibility, consistency and cost-effectiveness. There are no reports of PP7 occurring naturally in natural waters, and it was used previously to validate hollow fiber ultrafiltration because of its similarity in size (25 nm) and physico-chemical properties to poliovirus (Rajal et al., 2007).

The ultracentrifugation-based method resulted in a higher recovery rate of RV-A compared to the adsorption-elution method. Similar results were not obtained for PP7, demonstrating that the recovery efficiency depends on the physical and chemical properties of each virus, including specific density, morphology and membrane attachment patterns (Lewis and Metcalf, 1988; Nupen and Bateman, 1985). The high initial cost to acquire an ultracentrifuge has been a drawback preventing the widespread use of this technique. However, in laboratories with access to an ultracentrifuge, this method is an excellent alternative since it is robust and simple, and the tubes can be autoclaved and reused (Wyn-Jones and Sellwood, 2001).

The filters used in the adsorption-elution method can be clogged by the large quantity of debris that may be present in the sample, reducing the recovery rate (Guimarães et al., 2008; Victoria et al., 2010). Therefore, to prevent clogging, the sewage samples were diluted in 2 L of distilled water.

Both methods have been described as a useful tools to recover enteric viruses from sewage samples, allowing for detection using molecular methods, such as regular PCR and qPCR (Bofill-Mas et al., 2006; Guimarães et al., 2008; Ferreira et al., 2009; Fumian et al., 2010; Haramoto et al., 2006; Miagostovich et al., 2008; Pina et al., 1998; Rodríguez-Díaz et al., 2009; da Silva et al., 2007; Victoria et al., 2009; Villar et al., 2007).

To demonstrate the usefulness of the combination of the ultracentrifugation method and the NSP3 multiplex qPCR for investigating the natural occurrence of RV-A in wastewater, fourteen raw samples were analyzed. The results demonstrated the environmental dissemination of different G and P genotypes. As described previously, NSP3 qPCR provide a rapid and sensitive method for detection of RV-A and can detect different rotavirus G types, demonstrating its use for the detection of RV-A in environmental samples (Pang et al., 2004; Zeng et al., 2008). Moreover, according to Matthijnssens et al. (2008) NSP3 is the second-most conserved gene between the eleven genes of RV.

The multiplex qPCR assay evaluated in this study demonstrated a sensitivity similar to the monoplex assay and reduced the time and cost of detecting viruses in environmental samples. The availability of a sensitive method for viral quantification that includes an internal control, along with knowledge of the recovery efficiency of the concentration method, allows for a more accurate assessment of the potential presence of viruses in the environment.

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4.6 One year environmental surveillance of rotavirus specie A (RVA) genotypes in circulation after the introduction of the Rotarix® vaccine in Rio de Janeiro, Brazil

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One year environmental surveillance of rotavirus specie A (RVA) genotypes in circulation after the introduction of the Rotarix® vaccine in Rio de Janeiro, Brazil

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Objetivos:

- Avaliar a disseminação e concentração de RVA em uma ETE de grande porte (ETE Alegria - CEDAE-RJ) e determinar a diversidade de genótipos e a circulação de cepas vacinais.

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ABSTRACT

Rotavirus specie A (RVA) infection is the leading cause of severe acute diarrhea among young children worldwide. To reduce this major RVA health impact, the Rotarix® vaccine (GlaxoSmithKline, Rixensart, Belgium) was introduced in the Brazilian Expanded Immunization Program in March 2006 and became available to the entire birth cohort. The aim of this study was to evaluate the spread of RVA in the environment after the introduction of Rotarix® in Brazil. For this purpose, a Wastewater Treatment Plant (WTP) in Rio de Janeiro was monitored for one year to detect, characterize and discriminate RVA genotypes and identify possible circulation of vaccine strains. Using TaqMan® quantitative PCR (qPCR), RVA was detected in 100% (mean viral loads from 2.40×10^5 to 1.16×10^7 genome copies (GC)/L) of sewage influent samples and 71% (mean viral loads from 1.35×10^3 to 1.64×10^5 GC/L) of sewage effluent samples. The most prevalent RVA genotypes were P[4], P[6] and G2, based on VP4 and VP7 classification. Direct nucleotide sequencing (NSP4 fragment) and restriction enzyme digestion (NSP3) analysis did not detect RVA vaccine-like strains from the sewage samples. These data on RVA detection, quantification and molecular characterization highlight the importance of environmental monitoring as a tool to study RVA epidemiology in the surrounding human population and may be useful on ongoing vaccine monitoring programs, since sewage may be a good screening option for a rapid and economical overview of the circulating genotypes.

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1. Introduction

Rotavirus specie A (RVA) is the main etiological agent of viral gastroenteritis in infants throughout the world and is associated with significant mortality in developing countries, where over 600,000 deaths occur annually (Parashar et al., 2006). In developed countries, this virus remains a common cause of morbidity with significant economic burden (Charles et al., 2006; Parashar et al., 2006).

RVA belongs to the Reoviridae family, *Rotavirus* genus, and possesses a double-stranded RNA (dsRNA) genome with 11 segments that encode six structural (VP) and six non-

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structural proteins (NSP) (Estes and Kapikian, 2007). A widely used binary classification scheme has been established based on the two genes that codify the outer capsid proteins, VP4 and VP7, defining G (from VP7, glycoprotein) and P (from VP4, protease-cleaved protein) genotypes. Currently 27 G and 35 P genotypes are recognized (Abe et al., 2009; Solberg et al., 2009; Ursu et al., 2009; Matthijnssens et al., 2011); however, only five RVA G genotypes (G1–G4 and G9) and two P genotypes (P[8] and P[4]) are prevalent worldwide (Santos and Hoshino, 2005; Ursu et al., 2009).

RVA virions are shed in extremely high concentrations (up to 10^{10} virus/g) in the stool of infected children with acute gastroenteritis and can persist in the environment for long periods of time (Carter, 2005; Bosch et al., 2008). The features of the virions, including stability in aqueous environments and resistance to water treatment, may facilitate their transmission to humans via contaminated water (Ansari et al., 1991; Espinosa et al., 2008). Direct sewage discharge into environmental waters such as lagoons, rivers, beaches and coastal waters represents a public health problem mainly in developing countries. These contaminated waters have been broadly linked to the causation of several waterborne gastroenteritis outbreaks (Kukkula et al., 1997; Villena et al., 2003a; Schmid et al., 2005; Godoy et al., 2006). Despite the difficulty of determining the proportion of gastroenteritis cases due to contaminated water, it has been suggested that a significant percentage of the cases are related to the quality of the water (Bosch et al., 2008).

As RVA is one of the most important causes of mortality in infants worldwide, two equally safe and efficacious live oral rotavirus vaccines, G1P[8] RVA vaccine (RV1 – Rotarix®, GlaxoSmithKline, Rixensart, Belgium) and a pentavalent G1-G4 and P[8] RVA vaccine (RV5 – RotaTeq®, Merck and Co., Whitehouse Station, NJ, USA), were developed and are licensed for use in more than 100 countries worldwide (Jiang et al., 2010). The first one, RV1, was included in the Brazilian Expanded Immunization Program (PNI) in March 2006 and became available to the entire birth cohort.

The impact of this vaccine on the circulating RVA genotypes is unknown and difficult to predict, so continuous genotype surveillance is needed to identify the effects of the vaccine program on circulating strains, particularly on genotype prevalence and the emergence of uncommon strains. The monitoring of the viruses circulating in sewage from a wastewater treatment plant (WTP) has been described as an appropriate model to understand the spread of RVA in the population served by the WTP, as influents may contain viruses shed from patients with sporadic or asymptomatic cases (Haramoto et al., 2006; Bosch et al., 2008).

The main goal of this study was to evaluate the spread of RVA in the environment following the introduction of the Rotarix® vaccine in Brazil. For this purpose, a WTP located in Rio de Janeiro was monitored for one year to detect, quantify and characterize RVA genotypes and to investigate the possible presence of the vaccine strain in sewage samples. RVA genomes were investigated in samples collected from raw and treated sewage using Taqman® quantitative PCR (qPCR), and the P (VP4) and G (VP7) genotypes were characterized by nested PCR in a multiplex reaction (Gentsch et al., 1992; Gouvea et al., 1994). Protocols based on direct nucleotide sequencing (NSP4) and restriction enzyme digestion (NSP3) analysis (Rose et al., 2010) were applied to discriminate between wild-type and vaccine strains.

2. Materials and methods

2.1. *Wastewater treatment plant (WTP) sample collection*

Sewage samples were collected from an urban WTP located in the metropolitan area of Rio de Janeiro, Brazil. The WTP receives sewage from around 1.5 million inhabitants leaving in both the central and north zone of the city and is one of the largest in Brazil. Sewage treatment employs a secondary treatment (aerobic process: activated sludge) with an inflow mean of 2500 $L\ s^{-1}$. Initial sewage treatment is composed of grid separation and primary sedimentation (five primary settling tanks with a volume of 7700 m^3 each). There are four aeration tanks in parallel (volume: 11,500 m^3 per tank) with a capacity to treat 625 $L\ s^{-1}$ of effluent. Secondary sedimentation is performed in four secondary settling tanks (volume: 8800 m^3 per tank) with no chlorination before effluents are discharged into the water environment.

A total of 48 sewage samples were collected bi-monthly (15 day interval) from August 2009 to July 2010, 24 of them were collected from raw sewage (influent) and 24 from the final treated sewage (effluent). At each sampling point, 50 ml of sewage was collected in sterile plastic bottles, kept at 4 °C and transported to the laboratory for immediate analysis.

2.2. *Virus concentration*

Viruses were concentrated using the ultracentrifugation method as described by Pina et al. (1998). To avoid false negative results and to evaluate the presence of inhibitors, sewage samples were inoculated with 500 μl of an internal control (bacteriophage PP7) before the concentration assay, and the extracted RNA was diluted 10-fold (Fumian et al., 2010).

2.3. *Nucleic acid extraction, reverse transcription (RT) and quantitative PCR (qPCR)*

The viral dsRNA was extracted by the glass powder method (Boom et al., 1990), and the synthesis of cDNA was carried out by reverse transcription using a random primer (PdN6 – 50 A260 units – Amersham Biosciences, Chalfont St Giles, Buckinghamshire, UK). Multiplex qPCR to detect RVA and PP7 was performed as described previously (Fumian et al., 2010) using primers described by Zeng et al. (2008) and Rajal et al. (2007). RVA primers and probe were designed to target a highly conserved region of the non-structural protein 3 (NSP3), and the PP7 primers to amplify a region of the PP7 replicase gene. Both were synthesized by Applied Biosystems (CA, USA). qPCR was carried out using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, CA, USA). A standard curve (SC; 10^7, 10^5, 10^3 and 10^1 copies per reaction) was generated using 10-fold serial dilutions of a pCR2.1 vector (Invitrogen, USA) containing either the RVA NSP3 gene or the PP7 replicase gene.

2.4. VP4 and VP7 nested PCR amplification

Nested PCR was used for molecular characterization of RVA genotypes G and P, and it partially amplified VP7 and VP4 segments, respectively. In the first-round, RT-PCR was performed with VP7 and VP4 consensus primers 9con1–9con2 (Das et al., 1994) and 4con2–4con3 (Gentsch et al., 1992), respectively. Following the first-round, RVA G genotype classification was performed using specific primers for genotypes G1–G4, G5 and G9 (Das et al., 1994; Gouvea et al., 1994), and P genotype classification was carried out using primers for genotypes P[4], P[6] and P[8]-P[10], described by Gentsch et al. (1992).

2.5. RVA molecular characterization

To discriminate RVA wild-type G1P[8] from the vaccine strain, the NSP4 gene was amplified according to the protocol described by Cunliffe et al. (1998). Two nucleotide mutations after the first initiation ATG codon at positions 100 and 134 were observed when the NSP4 nucleotide sequence of the Rotarix® vaccine (patent number: PCT/EP2004/009725) was compared to sequences available in GenBank including reference strains. These two nucleotide shifts were used to classify RVA (data not shown).

The NSP4 PCR amplicons were purified and sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit® and an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The chromatograms were analyzed using BioEdit® (Hall, 1999). A phylogenetic dendrogram was constructed by the neighbor-joining method using a matrix of genetic distances established under the Kimura-two parameter model (Felsenstein, 1993) using MEGA V. 4.0 (Tamura et al., 2007). The robustness of each node was assessed by bootstrap analysis using 2000 pseudoreplicates. RVA NSP4 isolated from sewage samples was classified according to the most recent full genome-based classification proposed by Matthijnssens et al. (2011).

2.6. Cloning and restriction endonuclease analysis

To characterize vaccine strains, another protocol based on BspHI restriction endonuclease analysis of the NSP3 gene was performed as previously described (Rose et al., 2010). Prior to endonuclease restriction analysis, PCR amplicons generated by NSP3 amplification (Matthijnssens et al., 2006) were cloned into the PCR4-TOPO vector (Invitrogen, USA) following the manufacturer's recommendations.

2.7. Statistical analysis

The total frequency of detection obtained in WTP, in both influent and effluent samples, using qPCR assay was compared by using a chi-square test and Fisher's exact test at a significance level of 0.05. The same statistical analysis was performed to determine significant differences between VP4 and VP7 PCR detection in all of the 48 samples collected. Analysis of Variance (ANOVA) was performed to determine differences in mean levels of RVA, present in influent samples, during the four seasons (summer, fall, spring and winter), and a paired t-test was performed to verify differences between the mean levels of RVA in influent and effluent samples throughout the study. Statistical analyses were performed using GraphPad Prism® software version 5.

3. Results

3.1. Rotavirus A detection and quantification

The RVA genome levels and genotypes were determined in a one-year monitoring study from influent and effluent streams at a WTP located in Rio de Janeiro city, Brazil. Using qPCR, 41 out of 48 (85%) of the samples were positive, corresponding 100% (24/24) of influent and 71% (17/24) of effluent samples. The difference in the total frequencies of RVA detection (qPCR) in WTP was significant between influent and effluent samples ($p = 0.0042$, Chi-square; $p = 0.0047$, Fisher). Fig. 1 shows the monthly distribution of RVA genome copies (GC/L) and the standard deviation. For sewage influent, RVA concentrations ranged from 2.40×10^4 to 1.16×10^7 GC/L, and in effluent, positive sample concentrations, ranged from 1.35×10^3 to 1.64×10^5 GC/L. The differences in mean levels of RVA present in influent samples throughout the seasons were not significant (ANOVA/ Newman–Keuls Multiple

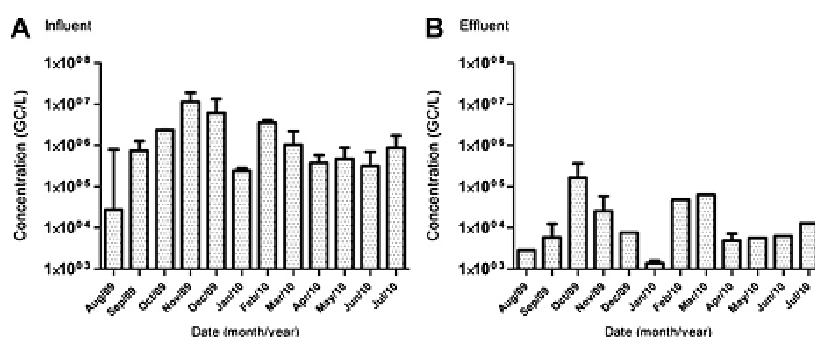

Fig. 1 – Monthly distribution of rotavirus specie A (RVA) in influent (A) and effluent (B) samples from a WTP in Brazil. The plots show the geometric monthly mean values (GC/L). The upper and lower bars show the standard deviations of the mean values.

Comparison Test), on the other hand, difference in the mean levels of RVA was significant between influent and effluent samples ($p = 0.041$, Paired t-test).

Table 1 summarizes the results obtained from the genomic amplification protocols used for detection, quantification (qPCR) and molecular characterization of RVA genes (RT-PCR for NSP4, VP4 and VP7). The RVA genotype G2 was detected in 100% (24/24) of influent samples, and the genotypes P[4] and P[6] were detected in 33% (8/24) and 25% (6/24), respectively. Effluent samples showed a lower RVA detection rate, with genotypes G2 and P[4] being detected in 25% (6/24) and 4% (1/24) of samples, respectively. Genotypes G1 and P[8] were not identified in the samples tested. The difference in the frequency of RVA detection using VP4 and VP7 PCR was significant ($p = 0.002$, Chi-square; $p = 0.004$, Fisher).

RT-PCR based on the NSP4 gene was able to detect viruses in 92% (22/24) and 21% (5/24) of influent and effluent samples, respectively. No evidence of inhibitors was observed as bacteriophage PP7, inoculated as an internal control in all 48 sewage samples, was detected in 100% of samples tested using a multiplex qPCR. PP7 viral titers recovered ranged from 3.4×10^5 to 1.6×10^4 GC per 500 µl of PP7 suspension.

3.2. *Rotavirus A strain characterization*

The sequence of gene segment 10 (encoding NSP4) of Brazilian waste samples were compared with NSP4 segments available in GenBank. The phylogenetic analysis of this segment classified the samples within two distinct genotypes: E1 genotype, with a single sequence (RJ-VA-550) and E2 genotype, in which 10 sequences clustered. Among the sequences that clustered in genotype E2, the sequence obtained from RJ-VA-575 sample clustered in a separate branch of the tree (Fig. 2). None of the 11 NSP4 sequences showed the two nucleotide mutations as in the vaccine pattern.

RVA NSP4 nucleotide sequences obtained in the present study were deposited at the National Center for Biotechnology Information (GenBank, http://www.ncbi.nlm.nih.gov/) under the accession numbers: RVA/Env-wt/BRA/RJ-VA-500/2009/GXP[X]: JF731369; RVA/Env-wt/BRA/RJ-VA-515/2009/GXP[X]: JF731370; RVA/Env-wt/BRA/RJ-VA-518/2009/GXP[X]: JF731371; RVA/Env-wt/BRA/RJ-VA-521/2009/GXP[X]: JF731372; RVA/Env-wt/BRA/RJ-VA-523/2009/GXP[X]: JF731373; RVA/Env-wt/BRA/RJ-VA-524/2009/GXP[X]: JF731374; RVA/Env-wt/BRA/RJ-VA-526/2009/GXP[X]: JF731375; RVA/Env-wt/BRA/RJ-VA-527/2009/GXP[X]: JF731376; RVA/Env-wt/BRA/RJ-VA-547/2009/GXP[X]: JF731377; RVA/Env-wt/BRA/RJ-VA-550/2009/GXP[X]: JF731378; and RVA/Env-wt/BRA/RJ-VA-575/2010/GXP[X]: JF731379.

In order to accurately determine the presence of the vaccine components in the environment, six influent samples with high viral loads (1.7×10^7–3.7×10^5 GC/L) were subjected to PCR amplification of the region of the genome encoding NSP3, and the resulting products were cloned. Forty-seven colonies were screened for the appropriate banding pattern after *BspHI* restriction endonuclease analysis, and none of them demonstrated the vaccine pattern. A Rotarix® NSP3 amplicon was analyzed in the same reaction as a positive control.

Table 1 – Rotavirus specie A (RVA) detection from influent (24) and effluent (24) sewage samples by quantitative (qPCR) and qualitative (NSP4, VP4, VP7) PCR protocols for genotyping.

Year	Month	Sewage influent				Sewage effluent			
		qPCR	NSP4	VP4	VP7	qPCR	NSP4	VP4	VP7
2009	Aug	+	–	–	G2	–	–	–	–
		+	+	P[4]	G2	+	–	–	–
	Sep	+	+	P[4]	G2	+	–	–	–
		+	+	–	G2	+	–	–	–
	Oct	+	+	P[6]	G2	+	–	–	G2
		+	+	P[4]	G2	+	+	P[4]	G2
	Nov	+	+	P[6]	G2	+	+	–	G2
		+	+	P[4]	G2	+	–	–	–
	Dec	+	+	P[4]	G2	+	–	–	–
		+	+	P[6]	G2	–	–	–	–
2010	Jan	+	+	P[6]	G2	+	–	–	–
		+	+	–	G2	+	–	–	–
	Feb	+	+	P[6]	G2	+	–	–	–
		+	+	–	G2	–	–	–	–
	Mar	+	+	–	G2	–	–	–	–
		+	+	–	G2	+	+	–	G2
	Apr	+	+	–	G2	+	+	–	G2
		+	+	–	G2	+	–	–	G2
	May	+	+	P[4]	G2	–	–	–	–
		+	+	–	G2	+	–	–	–
	Jun	+	–	–	G2	+	–	–	–
		+	+	P[4]	G2	–	–	–	–
	Jul	+	+	P[6]	G2	–	–	–	–
		+	+	P[4]	G2	+	–	–	–

+: Positive; and –: negative.

4. Discussion

In this study, an environmental approach was used to evaluate the circulation of RVA genotypes in the city of Rio de Janeiro, Brazil, which has the second largest population in the country. Samples from a large WTP were analyzed using a concentration method (ultracentrifugation) and molecular techniques to detect, quantify and characterize the detected viruses (Pina et al., 1998; Fumian et al., 2010). This type of approach has been extensively employed to obtain information on circulating viruses in populations throughout the world, independently of single reported cases or outbreaks, and to assess virus circulation causing asymptomatic infections (Bosch et al., 2008; Gajardo et al., 1995; Haramoto et al., 2006; Clemente-Casares et al., 2009; Fumian et al., 2010; Kamel et al., 2010; Prado et al., 2011). Besides revealing the predominant genotypes circulating in Rio de Janeiro, this monitoring strategy also aimed to investigate the presence of the attenuated G1P[8] RVA vaccine Rotarix® in the environment.

The combination of virus concentration and molecular detection methods was successfully employed, and the results showed a high level of RVA contamination in sewage samples. The high recovery rate of RVA (47%) from sewage samples (Fumian et al., 2010), using the ultracentrifugation method, was fundamental for the success in RVA recovering. Another study using this ultracentrifugation method to

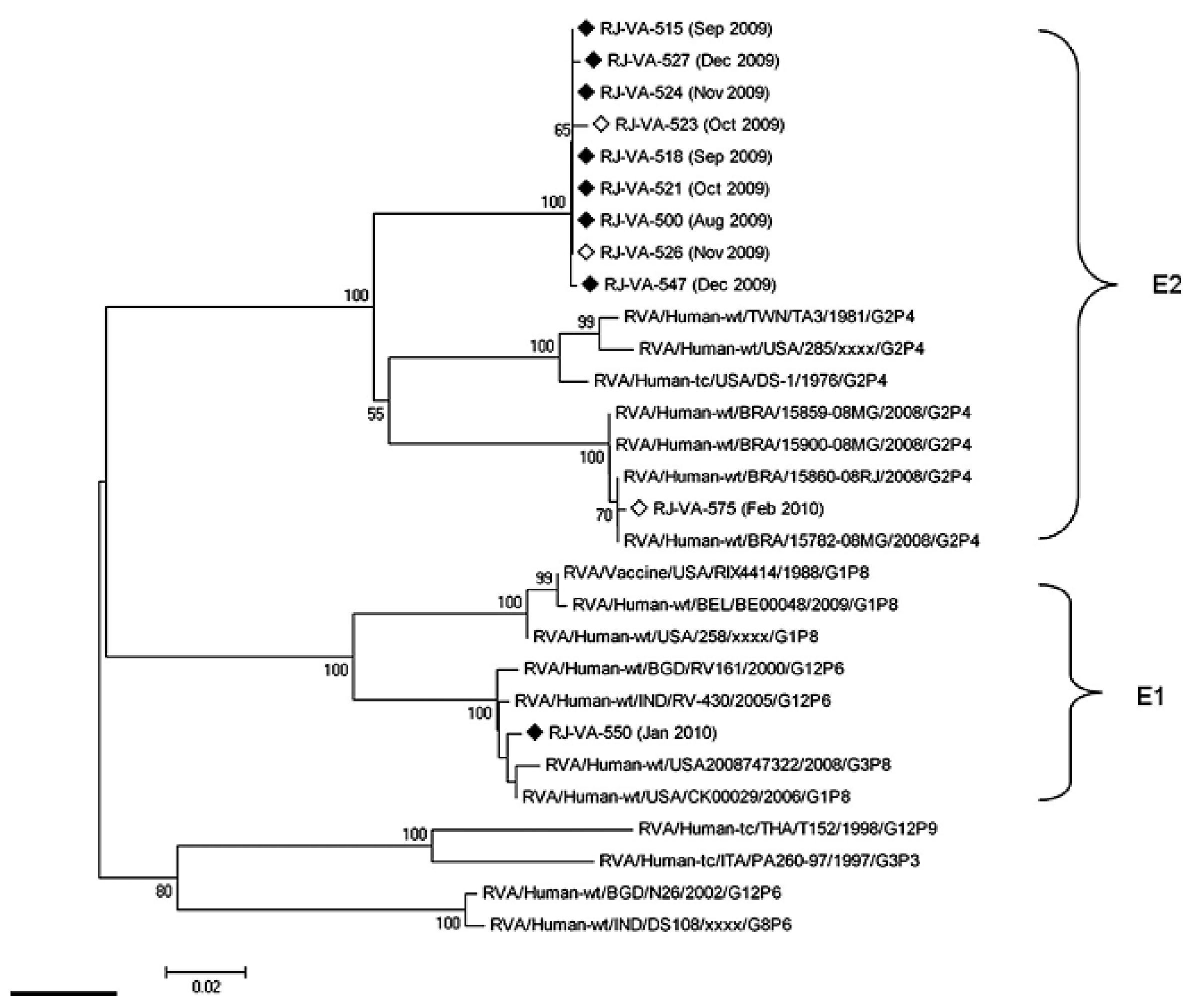

Fig. 2 – Phylogenetic dendrogram based on partial NSP4 nucleotide sequences of rotavirus A strains isolated from sewage samples in this study. All sequences obtained from GenBank are named according to Matthijnssens et al. (2011), and G and P genotypes are indicated at the right. The Brazilian environmental samples are marked with a filled diamond (influent samples) and an unfilled diamond (effluent samples). The scale bar at the bottom of the tree indicates distance. Bootstrap values (2000 replicates) are shown at the branch nodes and values lower than 50% are not shown.

recover RVA from domestic sewage and polluted water river samples demonstrated a high percentage of positive samples: 67% and 83% (Rodríguez-Díaz et al., 2009). Lower RVA detection rates have been observed when membrane-active charged filtration was used as a concentration method associated with organic or inorganic elution (Ferreira et al., 2009; Kamel et al., 2010).

A pattern of seasonality of RVA-induced gastroenteritis has been demonstrated in Latin American countries, including Brazil, based on a higher incidence of infection occurring in winter months (Kane et al., 2004; Carvalho-Costa et al., 2011). However, this differential distribution was not observed by the analysis of sewage samples during the monitoring period, suggesting a high level of virus shedding occurring throughout the year.

The average reduction of 2 logarithms in viral load observed in effluent samples demonstrates that WTPs play an important role in reducing environmental contamination. However, as demonstrated in this study, the persistence of such viruses in treated effluents and in other studies from different regions, highlights the importance of evaluating the efficiency of different types of treatments used by WTPs in viral load reduction (Bofill-Mas et al., 2006; Haramoto et al., 2006; da Silva et al., 2007; Meleg et al., 2008; La Rosa et al., 2010).

Despite the difficulties in associating virus infection to contact with contaminated water, the environmental dissemination of RVA, demonstrated by the high prevalence and concentration in the treated or untreated sewage samples, poses a risk to human health that must be considered and evaluated. Although the detection of nucleic acid does not

directly indicate the presence of infectious viruses, it is strongly suggestive of an infectious particle (Girones et al., 2010). Different studies have demonstrated that signals generated after RT-PCR amplification of viral genomes correlated well with infectivity or that a great part of viral nucleic acid recovered from environmental samples corresponded to infectious virus particles (Bhattacharya et al., 2004; Espinosa et al., 2008; Barrella et al., 2009).

The results obtained in this study regarding RVA dissemination, along with other studies conducted in developing countries, indicate RVA as a possible viral indicator of human fecal contamination in environmental samples, at least in countries where there is a high RVA prevalence (Ferreira et al., 2009; Miagostovich et al., 2008; Rodríguez-Díaz et al., 2009; Prado et al., 2011; Sdiri-Loulizi et al., 2010).

Data concerning virus genotyping provide significant epidemiological information necessary for the introduction and ongoing monitoring of vaccination programs (Villena et al., 2003b; Pinto et al., 2007; Bosch et al., 2008). The NSP4 segment analysis showed samples clustered with two genotypes. E1 sequence was close related to NSP4 from genotypes G1P[8], G3P[8] and G12P[6] and was probably associated with P [6] genotype detected. Within E2 genotype, nine samples formed a monophyletic group, and one sequence (RJ-VA-575) clustered in another group, including Brazilian G2P[4], isolated in 2008. The high detection frequency of E2 genotype, with G2 and P[4], is in agreement with trends described by Matthijnssens et al. (2011), where strains with a G2 and P[4] genotype presented an E2 profile and strains with a G1, G3, G12, P[6] and P[8] genotypes demonstrated an E1 profile.

The prevalence of G2 and P[4] genotypes in sewage samples is in agreement with the results obtained in a previous survey using clinical samples from acute infantile gastroenteritis cases in the municipality of Rio de Janeiro after Rotarix® introduction (Carvalho-Costa et al., 2009, 2011). RVA P[6] genotype detected in a lower prevalence than P[4] in sewage sample, reflects results obtained from clinical samples, showing that, in Brazil, the major circulation of G2P[4] and in a slight ratio, G2P[6] genotype. Data from the Laboratory of Comparative and Environmental Virology (LVCA), a Brazilian Regional Reference Laboratory for Rotaviruses, from 2009 to 2010, showed a higher percentage (78%) of RVA G2P[4] circulating in Rio de Janeiro when compared with other genotypes characterized as G4P[8] (6%); G9P[X] (6%); G2P[6], G2P[X] and G1P[X] (3%) (data not published). Although an increasing prevalence of genotype G2P[4] has also been reported in countries that have not established Rotarix® vaccination programs (Ferrera et al., 2007; Antunes et al., 2009), it is important to note that in a smaller WTP sewage monitoring program also conducted in Rio de Janeiro in 2005, before RVA vaccine introduction, G1 and P[8] were the most prevalent RVA genotypes detected (Ferreira et al., 2009). This change in the RVA genotypes prevalence profile could be explained by a natural genotypic fluctuation, although the role of the Rotarix® vaccine introduction cannot be ruled out (Gómez et al., 2011). In Australia, where both vaccine types are used, it was observed a higher prevalence of G2P[4] genotypes in states that used exclusively Rotarix® vaccine when compared with states that used Rotateq®, showing a higher prevalence of G3P[8] strains (Kirkwood et al., 2011). Another important aspect regarding the RVA vaccine is that Rotarix® prevents around 90% of severe gastroenteritis cases caused by G1P[8], as well as other partially heterotypic strains; however, it is less effective (45%) in preventing diarrhea caused by fully heterotypic G2P[4] strains (Ruiz-Palacios et al., 2006). Linhares et al. (2008), when evaluating Rotarix® efficacy against rotavirus gastroenteritis in a phase III study performed in Latin American infants, demonstrated a vaccine efficacy of 82% and 40% against G1P[8] and G2P[4], respectively.

Genotypes G1 and P[8] were not found in these sewage samples, showing that these genotypes are no longer circulating or are circulating at a very low level, reinforcing data obtained via surveillance of clinical specimens (Carvalho-Costa et al., 2009, 2011). The high shedding of RVA antigens (up to 10^{10} virus/g) from naturally infected individuals may restrict the detection of the vaccine strain that would be less prevalent in the environment. In a study conducted in South Africa during 2003–2004 to evaluate the safety, reactogenicity and immunogenicity of the Rotarix® vaccine, virus shedding was observed in healthy infants, ranging from 31% to 46% depending on the vaccination regimen used (Steele et al., 2010).

The cloning of the gene NSP3 followed by restriction enzyme analysis was an alternative attempt to increase the probability of vaccine strain detection, as cloning of the PCR products enables detection of genotypes that are at lower abundance in the environment. This methodology based on NSP3 gene amplification followed by *BspHI* digestion was previously described for discrimination of the Rotarix® vaccine (Rose et al., 2010).

Epidemiological and laboratory surveillances to assess vaccine effectiveness and vaccine impact are currently significant concerns (WHO, 2008). As Brazil was the first Latin American country to introduce universal rotavirus vaccination, the evaluation of vaccine performance to examine possible changing strain patterns of RVA in circulation is a priority in this country. Sentinel RVA surveillance in selected pediatric settings has been recommended as part of the immunization program in Latin America (Carvalho-Costa et al., 2009). Environmental surveillance, as conducted in this study by investigating RVA in sewage samples, could be an alternative approach to support clinical monitoring of RVA infection. This kind of surveillance would allow continuous investigation of the genotypes circulating in the WTP service area, providing an overview of the prevalent genotypes and possibly discriminating between RVA vaccine and wild strains.

5. Conclusion

(1) The high circulation of RVA in the population was measurable by environmental surveillance coupled with appropriate molecular tools.
(2) Wastewater surveillance demonstrated that genotypes G2 and P[4] were the most prevalent, reflecting a natural fluctuation of RVA genotypes or a consequence of Rotarix® vaccine introduction or even both.
(3) This is the first study concerning RVA detection and discrimination between vaccine and wild-type strains

from environmental samples carried out in a WTP and may assist clinical epidemiological studies that will be essential in the post-vaccination era.

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4.7 Assessment of burden of viral agents in an urban sewage treatment plant in Rio de Janeiro, Brazil

Assessment of burden of viral agents in an urban sewage treatment plant in Rio de Janeiro, Brazil

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Key Words:	Wastewater, Environmental health, Detection, Genotyping, Virus(es)

Objetivo:

- Determinar a disseminação e concentração viral de NoV, HAstV, HAdV e JCPyV em amostras de afluente e efluente da ETE Alegria a fim de se avaliar um marcador viral de contaminação humana em ecossistemas aquáticos.

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Assessment of burden of viral agents in an urban sewage treatment plant in Rio de Janeiro, Brazil

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ABSTRACT

Diarrhea remains a leading cause of mortality among young children in low and middle-income countries. Sewage discharge is considered the main source of viral contamination in aquatic environments, pointing the need of employ a virological marker, since it is really demonstrated that there is no correlation between the presence of viruses and fecal coliforms in the water. The aim of this study was to investigate the concentration of DNA and RNA viruses to evaluate a potential indicator of human contamination in wastewater. Influent and effluent samples were collected twice a month throughout a year. Viruses' detection was performed using quantitative PCR protocols and nucleotide sequencing was carried out for viruses genotyping. Human adenovirus (HAdV), polyomavirus JC (JCPyV) and rotavirus A (RVA) were the most prevalent viruses in influent samples (100%) with a viral load ranging from 10^6 to 10^5 genome copies per liter (gc l^{-1}). Norovirus (NoV) and human astrovirus (HAstV) were less prevalent with a viral load ranging from 10^4 to 10^3 gc l^{-1}. Quantitative data on the profile of viruses in wastewaters stress the high level of RVA environmental dissemination and address the potential of HAdV as a useful virological marker of virus contamination in aquatic environments. This study corroborates others performed in developed countries relating DNA viruses as good markers of human contamination, mainly HAdV, and emphasizes the importance to assess the impact of viral discharge on the environment that should be considered for health risk assessment studies.

Key words: Wastewater; environmental health; detection; genotyping; viruses; Brazil.

Introduction

Every day, approximately 5,000 young children die of diarrheal diseases worldwide, from easily preventable causes, and is estimated that 88% of deaths from diarrhea are caused by

contaminated water environments. Improved water and basic sanitation can cut this toll dramatically and simple, low-cost household water treatment has the potential to save further lives (WHO, 2004; WHO and UNICEF, 2005). Drinking water and sanitation coverage has progressed, but still represents a major challenge for health authorities, particularly in the developing world. In Brazil the access to a sewage collection network reaches half of the Brazilian population.

Raw sewage is a major carrier of disease causing agent and the discharge of natural raw or inadequately treated sewage effluents is the most common source of enteric viral pathogens in aquatic environments (Bosch, 1998). Then, the safe treatment of sewage has been appointed as a critical issue to the health of any community (Okoh et al., 2010).

Viruses are shed in high concentrations (up to 10^{11} copies g^{-1}) in feces or urine of infected patients (symptomatic and asymptomatic), and are transmitted primarily through the fecal–oral route, either directly from person to person or by contact with contaminated water or food (Bosch et al., 2008). Nowadays, molecular methodologies as quantitative polymerase chain reaction (qPCR) have been widely used to detect enteric viruses in aquatic environments, such as sewage and river water samples (Pusch et al., 2005; da Silva et al., 2007; Fumian et al., 2010; Haramoto et al., 2010; Prado et al., 2011).

Currently, microbiological water quality control and wastewater treatment systems performance are monitored by the use of bacterial indicator organisms (Okoh et al., 2010). On the other hand, reports have shown a lack of correlation between the presence or absence of bacterial indicators and viral contamination (Pusch et al., 2005; Carducci et al., 2008), while studies have been trying to identify a group of viruses that could be used as marker of human contamination in environment (Pina et al., 1998; Jiang et al., 2001; Fong and Lipp 2005; Bofill-Mas et al., 2006).

DNA viruses such as human adenovirus (HAdV) and polyomavirus JC (JCPyV) are usually more abundant than RNA viruses in water samples and have been recommended as

potential biological indicators for pollution of human origin and as a marker of viral contamination in water in developed countries (Bofill-Mas et al., 2000; Pina et al., 1998). However, there is a lack of data from developing countries on viruses' presence in wastewater or even concerning an organism index for virus contamination in the environment (Okoh et al., 2010). That´s why it is fundamental to characterize wastewater from countries located in tropical areas in order to provide data that will assist the evaluation of a single viral marker to be used worldwide.

This study presents data concerning DNA (HAdV and JCPyV) and RNA gastroenteric viruses as rotavirus species A (RVA), norovirus (NoV) and human astrovirus (HAstV) distribution in influent and effluent samples of a wastewater treatment plant (WTP) located in Rio de Janeiro, a metropolitan city of Brazil. Viruses' concentration protocol based on ultracentrifugation, qPCR and molecular methodologies for genotyping were used to detect, quantify and characterize viruses recovered from wastewater.

Materials and methods

Sewage samples

Sewage samples were collected from an urban WTP located in the metropolitan area of Rio de Janeiro, Brazil, previously described in details by Fumian et al. (2011). A total of 48 sewage samples were collected bi-monthly (15 day interval) from August 2009 to July 2010, 24 from raw sewage (influent) and 24 from the final treated sewage (effluent). At each sampling point, 50 ml of sewage was collected in sterile plastic bottles, kept at 4˚C and transported to the Laboratory of Comparative and Environmental Virology (LVCA) – Fiocruz, for immediate analysis.

Virus concentration and nucleic acid extraction

Virus particles were concentrated using the ultracentrifugation method previously described (Pina et al., 1998). Briefly, 42 ml of sewage was ultracentrifuged at 100,000 × *g* for 1 h at 4°C using a Beckman ultracentrifuge equipped with a type 35 rotor. Viral particles were resuspended in 3.5 ml of 0.25 mol l^{-1} glycine buffer (pH 9.5) and incubated on ice for 30 min. The solution was neutralized by the addition of 3.5 ml of 2x PBS (pH 7.2). The supernatant was clarified by centrifugation (12,000 × *g* for 15 min), and the viruses were finally recovered by ultracentrifugation at 100,000 × *g* for 1 h at 4°C in an SW41 rotor. Viral particles were resuspended in 400 µl of 1× PBS pH 7.2 and processed immediately for nucleic acid extraction or stored at -80°C until virus DNA/RNA extraction.

Viral nucleic acid was extracted by the glass powder method (Boom et al., 1990), and for RVA, NoV and HAstV, the synthesis of cDNA was performed by reverse transcription using a random primer (PdN6 – 50 A260 units – Amersham Biosciences, Chalfont St Giles, Buckinghamshire, UK).

Virus detection and quantification

Viruses' detection was performed using a TaqMan® qPCR. Primers and probes sequences and final concentrations, annealing temperature conditions and references for quantification and characterization of each viral group are shown in Table 1. The quantification was carried out in volume of 25 µl, containing five µl of the DNA extract or prepared cDNA mixed with 12.5 µl of TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA). Optical plate containing the mixtures were placed into an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) on the following conditions: incubation at 50°C for 2 min to activate UNG, initial denaturation at 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 56-60°C, depending on virus type, for 1 min. A 10-fold serial dilution of plasmid DNA containing the target sequences (specific to each virus group) was used to generate a standard curve between log standard concentrations (10^6–10^1 copies

per reaction) and threshold (Ct) value. Amplification data were collected and analyzed with Applied Biosystems 7500 Software® v2.0 (Applied Biosystems, Foster City, CA). Samples signals that cross the threshold line, presenting a characteristic sigmoid curve, were regarded positive. Virus concentration results are present as genome copies per liter (gc l^{-1}) of sewage samples.

Nucleotide sequencing and phylogenetic analysis

The molecular characterization of HAdV, JCPyV, NoV and HAstV was performed by nucleotide sequencing and phylogenetic analysis, using the amplicons generated from the hexon gene, an intergenic region fragment, the polymerase gene and VP1 protein, respectively (Table 1).

The amplicons generated in the RT-PCR and nPCR were purified with the QIAquick PCR Purification Kit® or QIAquick Gel Extraction Kit® (Qiagen, CA, USA), according to the manufacturer's instructions. PCR products were sequenced using an ABI Prism® 3100 Genetic Analyzer and Big Dye® Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA) in both directions.

Nucleotide sequences were edited and aligned with BioEdit® Sequence Alignment Editor (Hall, 1999). The sequences were compared with their prototypes as well as with other sequences of the National Center for Biotechnology Information (NCBI/ GenBank) (http://www.ncbi.nlm.nih.gov/). Phylogenetic trees were constructed using MEGA v.4.0 (Tamura et al., 2007) with the neighbor-joining method, using genetic distance corrected by the Kimura two-parameter model with 2,000 pseudoreplicates.

Methods and results regarding RVA quantification and genotyping were published previously in a study that evaluated the presence of RVA vaccine virus in the environment (Fumian et al., 2011). It was included to complete the list of viruses with epidemiological

importance in the country and to comparatively assess the viral load present in sewage samples.

For all molecular procedures, four separate rooms were used to avoid cross contamination of samples. The samples were analyzed in duplicated and specific positive and negative controls (clinical samples for each virus type and RNA/DNA-free water) were used in all procedures.

Statistical analysis

The frequency of detection, of each tested virus obtained in 48 samples of wastewater using qPCR assay was compared by using a chi-square test and Fisher's exact test at a significant level of 0.05. The occurrence of HAdV, JCPyV and RVA in the effluent was analyzed for significant differences. Statistical analyses were performed using GraphPad Prism® software version 5.

Results

Virus detection and concentration in influent and effluent samples

Table 2 shows results of viruses´ detection from influent and effluent samples obtained in the WTP located in an urban area of the city of Rio de Janeiro. The results showed a higher detection of HAdV, JCPyV and RVA when compared to NoV and HAstV ($p<0.05$, Chi-square). The detection of HAdV was significantly higher when compared to JCPyV ($p=0.02$, Fisher), but not with RVA ($p=0.79$, Chi-square). There was no difference in the detection of NoV and HAstV ($p=0.08$, Fisher).

Mean monthly concentration of HAdV, JCPyV, RVA, NoV GII and HAstV detected in wastewater samples are illustrated in Figure 1. HAdV concentration ranged between 5.23×10^5 and 7.04×10^6 gc l^{-1} (mean of 2.97×10^6 gc l^{-1}) and between 7.40×10^3 and 6.48×10^4 gc l^{-1} (mean of 2.55×10^4 gc l^{-1}) in influent and effluent samples, respectively.

The concentration of JCPyV in influent samples ranged between 3.26 x 10^4 and 2.11 x 10^6 gc l^{-1} (mean of 5.98 x 10^5 gc l^{-1}). In effluent samples, JCPyV DNA genome could be detected in four samples during the studied period. The concentration ranged between 2.54 x 10^3 and 4.28 x 10^3 gc l^{-1} (mean of 3.31 x 10^3 gc l^{-1}).

RVA concentration obtained previously in influent samples ranged between 2.74 x 10^4 and 1.68 x 10^7 gc l^{-1} (mean of 2.36 x 10^6 gc l^{-1}). In effluent samples, RVA concentration ranged between 1.11 x 10^3 and 3.04 x 10^5 gc l^{-1} (mean of 3.24 x 10^4 gc l^{-1}).

NoV GII concentration ranged between 1.50 x 10^4 and 2.03 x 10^5 gc l^{-1} (mean of 6.75 x 10^4 gc l^{-1}) in influent samples. In effluent samples NoV GII concentrations ranged between 2.19 x 10^4 and 1.66 x 10^4 gc l^{-1}, respectively. NoV GI was not detected in the tested samples.

HAstV mean virus concentration ranged between 6.23 x 10^2 and 2.71 x 10^3 gc l^{-1} (mean of 1.81 x 10^3 gc l^{-1}) and were not detected in effluents samples.

The results obtained by qPCR analysis demonstrated that HAdV and RVA were found in both raw and treated sewage year-round in high concentrations. On the other hand, JCPyV that was detected throughout the year in raw sewage was detected in four effluent samples only. JCPyV frequency detection in effluent samples was significantly lower compared to HAdV (p=0.001, Chi-square) and RVA (p=0.002, Chi-square).

Molecular characterization

HAdV, JCPyV, NoV and HAstV nucleic acids from the amplicons obtained by nPCR were sequenced and confirmed by a nucleotide sequence Basic Local Alignment Tool (BLAST) search. RVA molecular characterization was published elsewhere (Fumian et al., 2011).

JCPyV strains showed a high nucleotide identity with each other, ranging from 98.9 to 100%. The phylogenetic tree obtained with JCPyV sequences and JCPyV prototypes available in the GenBank is show in Figure 2. All the sequences detected in this study clustered with genotype type-3 (AF2), belonging to East Africa origins.

The phylogenetic tree generated with HAdV sequences revealed that all sequences clustered with HAdV type 41, showing a nucleotide homology between 95.3 and 98.9% with prototype sequence of HAdV41 (GenBank accession number X51783) (Figure 3).

Sequences of NoV amplified from the polymerase region (ORF-1) were classified as genogroup II (GII), and sequences of HAstV amplified from VP1 protein were classified as genotype 1 (HAstV-1).

All sequences obtained were deposited at the National Center for Biotechnology Information (GenBank, http://www.ncbi.nlm.nih.gov/) under the following accession numbers: HAdV: JN654703 – JN654713; JCPyV: JN644470 – JN644480; NoV: JN654714 – JN654718; and HAstV: JN799266 – JN799271.

Discussion

Sewage discharge is considered the main source of microbiological contamination in several aquatic environments becoming enteric viruses, presented in high concentration and highly stable in those environments as important contaminants of human exposure throughout contaminated water (Okoh et al., 2010). Raw sewage discharge into waters bodies, with the subsequent adverse effects on human health is not uncommon in developing world. In countries of South America where in general sewage network is still not sufficient, common pollution problems associated with urban sewage discharges (Rodríguez-Díaz et al., 2008) have contributed to morbidity and mortality from acute diarrhea.

In the present study, DNA viruses as HAdV and JCPyV presented high detection and concentration in raw sewage samples along the year following a similar pattern for DNA viruses worldwide corroborating those viruses to be good viral markers of human contamination (Bofill-Mas et al., 2006; Carducci et al., 2008; Katayama et al., 2008; Fong et al., 2010; Fumian et al., 2010; La Rosa et al., 2010; Schlindwein et al., 2010; Kokkinos et al., 2011).

JCPyV detection suffered an important reduction after processed by the WTP as observed in a previous study (Fumian et al., 2010). A lower detection (17%) of JCPyV in treated sewage samples compared with HAdV detection (75%) could be explained by a one log lower concentration of JCPyV in influent samples. Virus-negative samples were calculated to contain viral genomes with a concentration lower than 2.0 x 10^3 and 5.8 x 10^3 gc l^{-1}, for DNA and RNA virus, respectively, considering the detection limit of 5 gc per reaction by qPCR. Physical removal process, like activated sludge used in the WTP studied, able to remove about 90-99% of virus load (between one and two logs) of the wastewater (Ueda et al., 2004), reduced JCPyV concentration to a non-detectable level, lower than 2 x 10^3 gc l^{-1}, in effluent samples. Prado et al. (2011) reported 58% of hepatitis A virus (HAV) detection with mean concentrations of 6.5 x 10^5 gc l^{-1} in raw sewage samples obtained at the same WTP. Previous studies have demonstrated the lower concentration of JCPyV in water samples, compared with HAdV (Bofill-Mas et al., 2006; Haramoto et al., 2010). Other studies presented preferable features of HAdV as an indicator of viral contamination in water such as high stability in the environment, high resistant to UV disinfection, human specificity and worldwide occurrence throughout the year, with fecal excretion of gastroenteric and non-gastroenteric serotypes (Thompson et al., 2003; Flomenberg, 2005; Bofill-Mas et al., 2006; Girones, 2010).

However, in this study it was also demonstrated the importance of a well characterization of the local wastewater. The concentration of RVA in raw sewage was quite similar to concentration of HAdV and JCPyV revealing those viruses as an important environmental contaminant in this region as also observed in other developing countries such as Venezuela and China (Rodríguez-Díaz et al., 2008; He et al., 2011). The high concentration of RVA in the environment even after the introduction of a RVA vaccine is remarkable (Rotarix®, GlaxoSmithKline, Rixensart, Belgium) becoming available to the whole birth cohort in Brazil from 2006.

Single strand RNA viruses, as NoV and HAstV, were detected in lower concentration in sewage samples, as observed in a previous survey carried out in a smaller WTP in the city of Rio de Janeiro (Guimarães et al., 2008; Victoria et al., 2010), as well as in others similar studies performed in Greece and China (Kokkinos et al., 2011; He et al., 2010). Arraj et al. (2008) have not detected NoV and enteroviruses for the 18 months period of the study in a WTP, relating the negative results to the small sample volume, to the sensitivity of the detection methods or to local epidemiological circumstances. However, results obtained from five WTP in Italy showed a high prevalence of NoV in wastewater samples showing a different pattern of waterborne viruses' circulation according with the profile of the population studied (La Rosa et al., 2010). In Venezuela, it was demonstrated a high detection rate of NoV (75%), followed by HAstV and RVA (67%) and HAdV (50%) in an urban sewage (Rodrígez-Díaz et al., 2008). Together, these results reinforce the importance of studies in different cities and countries worldwide to obtain an epidemiology profile of viruses in wastewater samples.

The excretion patterns of RVA, NoV and HAstV and their dissemination as environmental contaminants in water was described as depending on seasonality, specific geographic area of study (high or low prevalence of a specific pathogens) and to the potential presence of outbreaks in the population (Girones et al., 2010). The use of different protocols could also cause mismatch results between unrelated studies. Bofill-Mas et al. (2006) compared two qPCR methods for their suitability in the detection and quantification of HAdV in waste samples and concluded that results were significantly different between both methods.

RVA data was published previously (Fumian et al., 2011) and was included in this study for comparative analysis of the concentration of the main viruses responsible for acute gastroenteritis. Viral average concentrations above 10^3 gc l^{-1} were demonstrated for all viruses, as expected in influent samples. However, the high concentration of RVA (mean of

10^6 gc l^{-1}) detected along the year, similar with results set obtained for HAdV, highlight and suggest the potential of RVA as virological marker in some geographic areas.

The environmental approach of detection and molecular characterization of virus in WTP's influent samples contributes with epidemiological surveillance studies providing information on genotypes circulating in a particular geographic region. Regarding the prevalence of major genotypes of virus responsible for acute gastroenteritis circulating in Rio de Janeiro, the results found in this study are in agreement with data from LVCA (a Regional Reference Laboratory of viral gastroenteritis) obtained from clinical samples at the same geographic area (Filho et al., 2007; Victoria et al., 2007; Ferreira et al., 2010; Carvalho-Costa et al., 2011) and also with previous studies performed in a small treatment plant in the same geographical region (Guimarães et al., 2008; Ferreira et al., 2009; Victoria et al., 2010).

Concerning DNA viruses, data from HAdV characterization corroborated previous studies demonstrating that specie F of HAdV (types 40 and 41) is the most prevalent specie found in aquatic environments (Ko et al., 2005; Haramoto et al., 2010; Fong et al., 2010; Cantalupo et al., 2011; Kokkinos et al., 2011) and JCPyV with results observed in a study performed in a smaller WTP in Rio de Janeiro (Fumian et al., 2010). Brazil has a continental area and further studies with samples from other states should be carried out to clarify if this African origin pattern of JCPyV is a characteristic of Rio de Janeiro's population or from Brazilian's population.

The results obtained in this study shows that WTP could be efficient at removing approximately two logs units of viruses present in raw sewage and revealed throughout quantitative data the high level of HAdV, JCPyV and RVA environmental dissemination, addressing the potential of HAdV as a useful virological marker of virus contamination. The viral load reduction performed by WTP should be highlighted since sewage treatment is one of the most effective systems to reduce the presence and concentration of viruses and others microbiological agents circulating in aquatic environments generated by human sewage

discharge. However, our data together with previous one that showed infectious HAdV in a WTP after sewage treatment with activated sludge and final chlorination (Carducci et al., 2008) stressed the need for a virological marker as a parameter to be used for water quality control.

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Table 1. Oligonucleotide primers and probes sequences, amplification conditions and references used for detection and molecular characterization of enteric virus.

Virus	Names	Sequence (5'- 3')	Annealing temperature (^{0} C)	Final concentration (nM)	Reference
HAdV[a]	HAdF	CWTACATGCACATCKCSGG		900	Hernroth et al.,
	HAdR	CRCGGGCRAAYTGCACCAG	60	900	(2002)
	HAdP1[c]	CCGGGCTCAGGTACTCCGAGGCGTCCT		225	
JCPyV[a]	JE3F	ATGTTTGCCAGTGATGATGAAAA		500	Pal et al.,
	JE3R	GGAAAGTCTTTAGGGTCTTCTACCTTT	60	500	(2006)
	JE3P[c]	AGGATCCCAACACTCTACCCCACCTAAAAAGA		200	
NoV[a]	COG2F	CARGARBCNATGTTYAGRTGGATGAG		600	Kageyama et al.,
	COG2R	TCGACGCCATCTTCATTCACA	56	600	(2003)
	RING2[c]	TGGGAGGGCGATCGCAATCT		250	
HAstV[a]	Av1	CCGAGTAGGATCGAGGGT		120	Le Cann et al.,
	Av2	GCTTCTGATTAAATCAATTTTAA	55	120	(2004)
	AvP[c]	CTTTTCTGTCTCTGTTTAGATTATTTTAATCACC		100	
HAdV[b]	Hex1deg	GCCSCARTGGKCWTACATGCACATC	55	500	
	Hex2deg	CAGCACSCCICGRATGTCAAA		500	Allard et al.,
	Hex3deg	GCCCGYGCMACIGAIACSTACTTC	55	500	(2001)
	Hex4deg	CCYACRGCCAGIGTRWAICGMRCYTTGTA		500	
JCPyV[b]	EP1A	TGAATGTTGGGTTCCTGATCCCACC	59	500	
	EP2A	ACCCATTCTTGACTTTCCTAGAGAG		500	Bofill-Mas et al.,
	P1A	CAAGATATTTGGGACACTAACAGG	59	500	(2000)
	P2A	CCATGTCCAGAGTCTTCTGCTTCAG		500	
NoV[b]	JV13I	TCATCATCACCATAGAAIGAG	45	440	Vennema et al.,
	JV12Y	ATACCACTATGATGCAGAYTA		440	(2002)
HAstV[b]	Mon269	CAACTCAGGAAACAGGGTGT	50	400	Noel et al.,
	Mon270	TCAGATGCATTGTCATTGGT		400	(1995)

[a] Primes and probes used for detection (qPCR); [b] Primes used for molecular characterization; [c] 6-Carboxyfluorescein (FAM) as the reporter dye is coupled in the 5´end of the oligonucleotide, and 6-carboxy-tetramethylrhodamine (TAMRA) as the quencher dye is coupled in the 3´end of the oligonucleotide;

Table 2. Percentage of positive samples detected by quantitative PCR in influent and effluent from the wastewater treatment plant located in Rio de Janeiro, Brazil.

	Viruses				
Waste samples (n)	**HAdV**	**JCPyV**	**RVA***	**NoV GII**	**HAstV**
	Number of positive samples (%)				
Influent (24)	24 (100%)	24 (100%)	24 (100%)	13 (55%)	7 (29%)
Effluent (24)	15 (75%)	4 (17%)	14 (71%)	2 (8%)	0 (0%)
Total (48)	39 (81.6)	28 (58.3)	38(79.2)	15(31.3)	7 (14.6)

*RV-A results previously published (Fumian et al., 2011).

Figure 1 Mean monthly concentration (genome copies per liter (gc l^{-1}) of HAdV, JCPyV, RVA, NoV GII and HAstV in influent and effluent samples collected during one year in WTP located in Rio de Janeiro, Brazil.

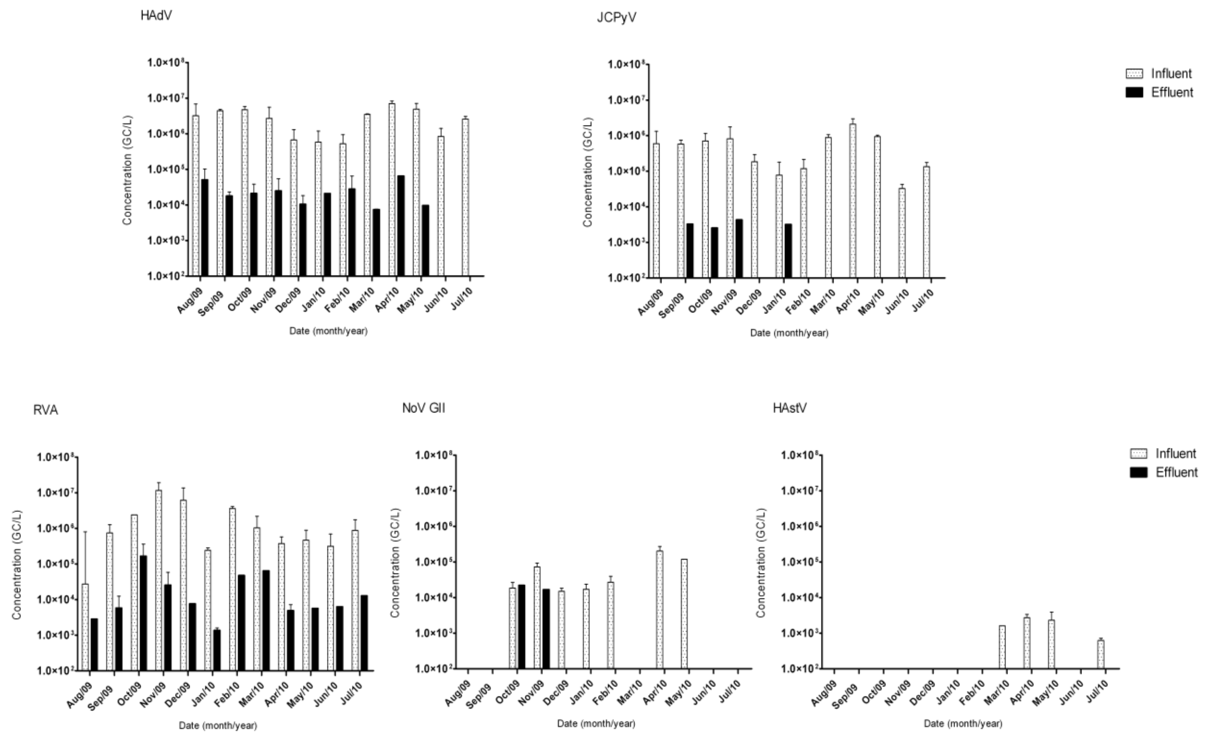


Figure 2 Phylogenetic dendrogram based on the partial intergenic region of 11 sequenced JCPyV strains. All sequences obtained from GenBank are named according to Pavesi (2003), and the corresponded accession numbers are indicated at the right. Brazilian environmental samples are marked with a filled diamond. The scale bar at the bottom of the tree indicates distance. Bootstrap values (2000 replicates) are shown at the branch nodes and values lower than 50% are not shown.

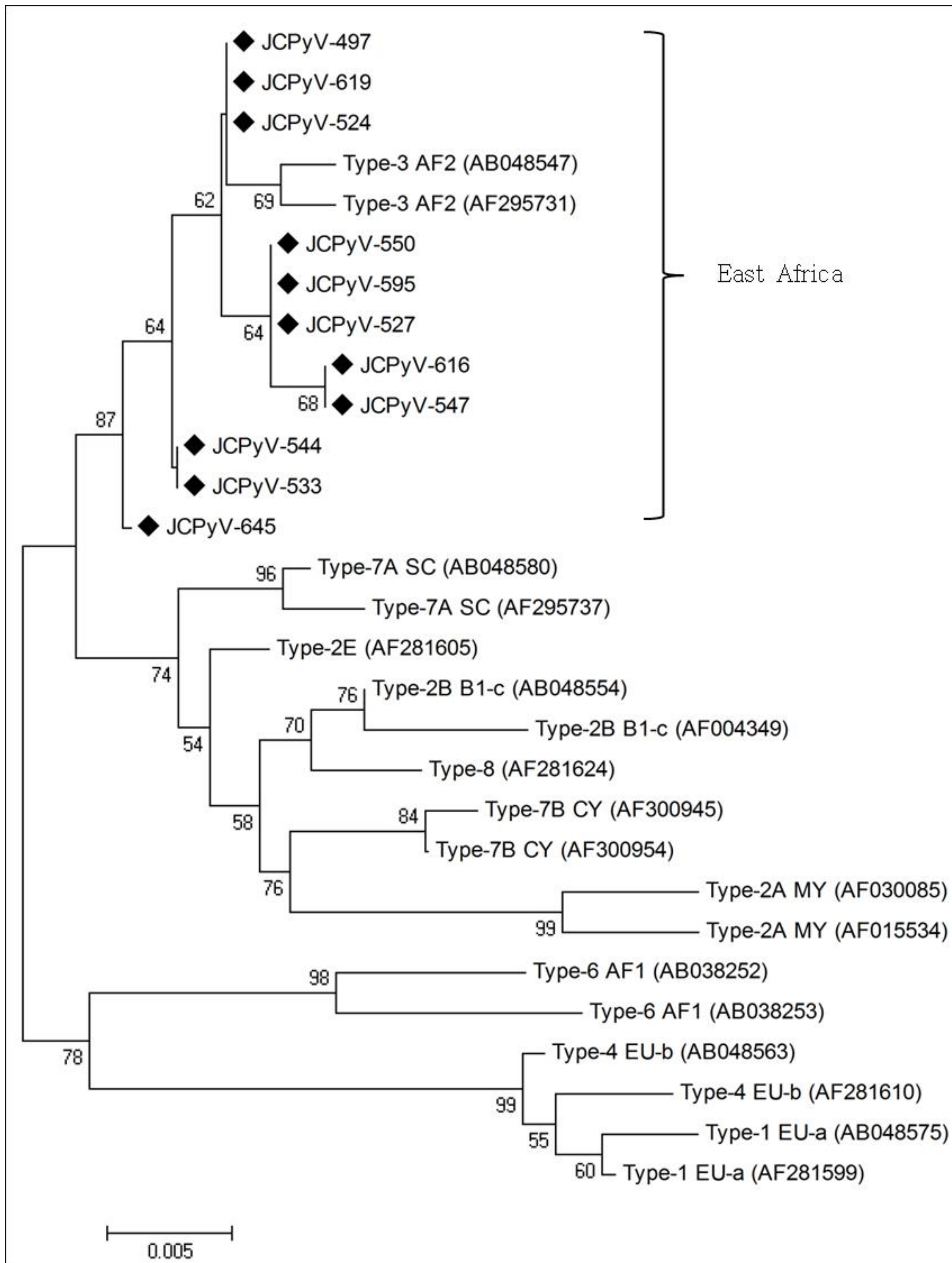
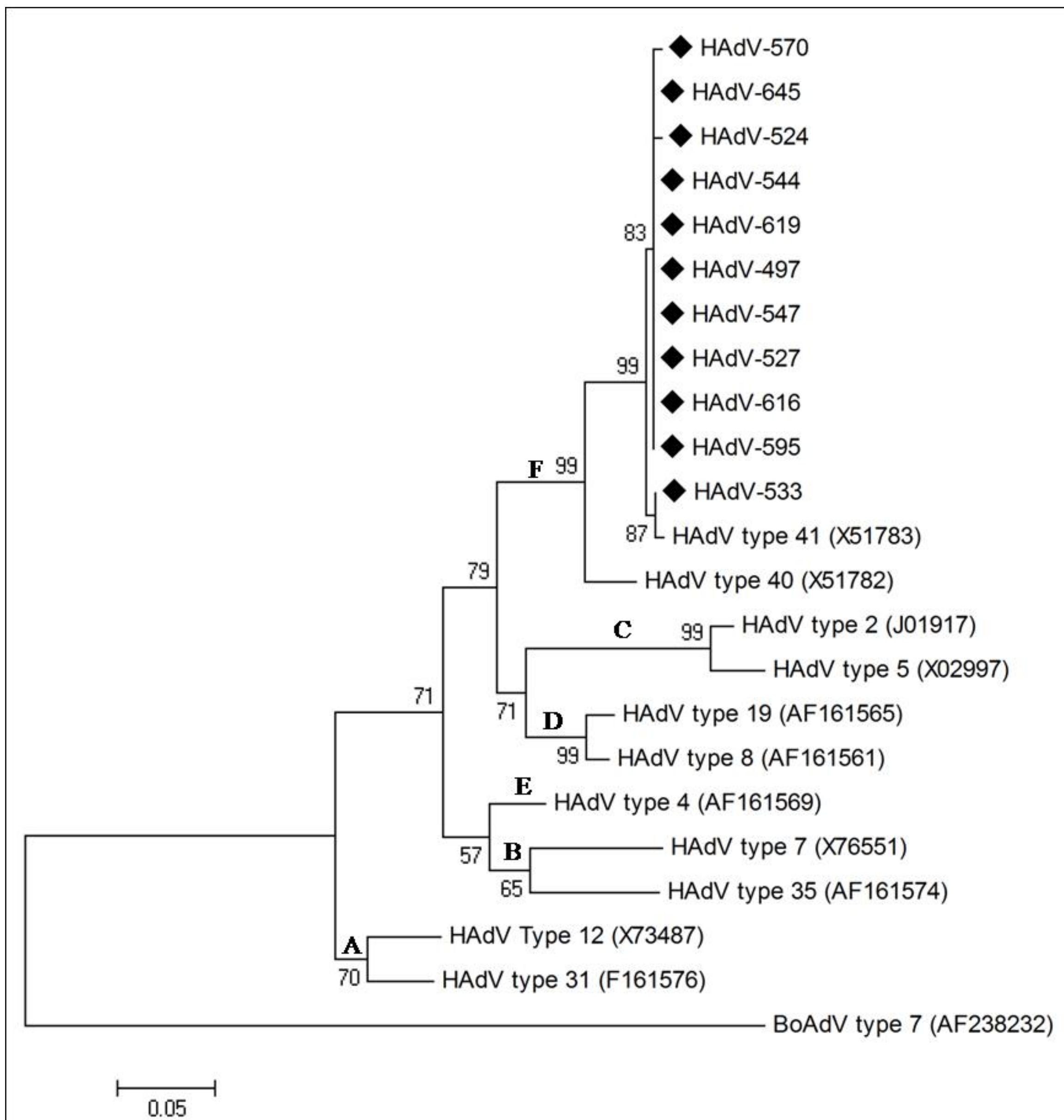


Figure 3 Phylogenetic tree of 11 HAdV (marked with a filled diamond) isolated from influent samples with prototypes sequences between the 253-bp sequences within the hexon gene. The six human adenovirus species (A-F) are indicated with letters. Bovine adenovirus type 7 sequence available in GenBank was used as out group. The scale bar at the bottom of the tree indicates distance. Bootstrap values (2000 replicates) are shown at the branch nodes and values lower than 50% are not shown.



5. DISCUSSÃO

Doenças de veiculação hídrica estão entre uma das causas mais comuns de morte no mundo e afetam especialmente países em desenvolvimento. Estima-se que em torno de 25% dos leitos hospitalares no mundo sejam ocupados por pacientes com doenças de veiculação hídrica (Straub & Chandler 2003). Surtos de doenças virais entéricas de veiculação hídrica têm sido descritos em muitos países e a lista de microorganismos potencialmente patogênicos continua aumentando (Leclerc et al. 2002; Straub & Chandler 2003).

O crescente número de relatos de surtos de GA de veiculação hídrica associados a agentes virais demonstra a importância do estudo dos níveis de contaminação viral em diferentes ecossistemas aquáticos complementando estudos epidemiológicos ou mesmo fornecendo dados originais, onde estudos com amostras clínicas ainda não tenham sido realizados (Maunula et al. 2005; Hewitt et al. 2007; Okoh et al. 2010).

A detecção, quantificação e caracterização molecular de vírus em amostras de águas residuárias da cidade do Rio de Janeiro tiveram como objetivo demonstrar a presença destes agentes em efluentes e afluentes de ETEs. Com uma abordagem ambiental foi realizada a vigilância laboratorial dos genótipos circulantes na população, uma vez que os vírus detectados em amostras ambientais de esgoto refletem o padrão de circulação destes na população. A eficiência de remoção viral determinada pela concentração da carga antes e após o tratamento utilizado por determinada ETE também foram avaliados. A estratégia para o desenvolvimento deste estudo foi a realização de monitoramentos em duas ETEs localizadas na cidade: ETE Fiocruz (2005) e ETE Alegria (2009 – 2010), de modo que os resultados obtidos serão discutidos de acordo com os objetivos alcançados em cada ETE.

5.1 Detecção de vírus na ETE Fiocruz – Estabelecimento e avaliação de metodologias de concentração, detecção e quantificação viral

Para avaliação da presença de diferentes agentes virais (RVA, HAstV, NoV e JCPyV) em amostras de águas residuárias da ETE Fiocruz foi utilizada a metodologia desenvolvida por Katayama e colaboradores (2002). Esta metodologia de concentração viral é baseada na adsorção-eluição dos vírus presentes em amostras de águas ambientais à membrana de ésteres de celulose carregada negativamente, seguida da eluição dos vírus da membrana e reconcentração por ultrafiltração. Esta metodologia utiliza soluções inorgânicas para eluição viral e tem sido descrita como alternativa a métodos que utilizam membrana carregada positivamente, que realizam a eluição dos vírus da membrana com extrato de carne, descrito

como inibidor das reações moleculares e tem sido utilizada para a detecção de diversos agentes virais presentes em diferentes tipos de ecossistemas aquáticos (Katayama et al. 2002; Haramoto et al. 2005, 2006).

A ETE Fiocruz localiza-se na cidade do Rio de Janeiro, dentro do Campus de Manguinhos, Fundação Oswaldo Cruz (Fiocruz). É uma estação de tratamento secundário por lodos ativados e aeração prolongada. A população atendida de projeto é de 8000 habitantes, perfazendo uma vazão total de 512m³/dia. Atualmente, em função da precariedade da rede coletora, trata em torno de 30% do volume de projeto, sendo que o efluente tratado é lançado no Rio Faria Timbó, que margeia o terreno do Campus.

5.1.1 Astrovírus (Artigo 4.1)

Para detecção de HAstV foram utilizado as técnicas de amplificação genômica qualitativa e quantitativa (RT-PCR e qPCR). A técnica de PCR convencional (Noel et al. 1995) foi utilizada para detecção de HAstV. Neste trabalho, foi realizado o estabelecimento e implementação a quantificação viral baseada na metodologia TaqMan® previamente descrita (Le Cann et al. 2004).

Oito amostras do total de 48 amostras coletadas foram positivas para HAstV (16,7%), sendo que quatro de afluente e quatro de efluente. Sete amostras foram detectadas PCR convencional e uma amostra foi detectada utilizando o qPCR. Neste estudo foi avaliado o percentual de recuperação da metodologia de adsorção-eluição para concentração e recuperação de HAstV pela realização de experimentos experimentais de contaminação viral em amostras de afluente e efluente. A eficiência de recuperação de HAstV foi de 4,2% e 4,3% para afluente e efluente, respectivamente. Este baixo percentual de recuperação pode ser uma das explicações para o baixo número de amostras positivas nas amostras analisadas.

Outros estudos de detecção de HAstV em águas residuárias encontraram percentual mais elevado de amostras positivas, variando de 43-100% e 82,3% em amostras de afluentes e efluentes, respectivamente (Nadan et al. 2003, Le Cann et al. 2004, Meleg et al. 2006).

Uma consideração importante que pode influenciar na diferença do percentual de detecção entre diferentes estudos é a prevalência de infecções por HAstV na população no período de coleta do estudo e se a ETE em estudo recebe afluente de algum hospital pediátrico e/ou creches, onde as infecções por HAstV são mais prevalentes nessa faixa etária. A escolha de volumes da amostragem, metodologias de concentração viral e métodos de amplificação são essenciais para a real determinação da prevalência de agentes virais em

matrizes aquáticas. Métodos com baixa eficiência de recuperação viral podem subestimar a real prevalência de determinado agente viral em uma matriz aquática estudada.

Rodriguéz-Díaz e colaboradores (2009), utilizando nested-PCR para detecção de HAstV em amostra de água de esgoto e rio obteve um percentual de detecção de 67% (8/12) e 89% (16/18), respectivamente. O método de concentração viral utilizado (ultracentrifugação), somado a utilização da nested-PCR, mais sensível para detecção viral, pode ter influenciado para a maior detecção de HAstV nas amostras de esgoto analisadas. O método de concentração viral utilizado nas amostras coletadas na ETE Fiocruz foi umas das causas relacionadas a baixa detecção obtida para HAstV nestas amostras.

Este foi o primeiro trabalho de detecção de HAstV em amostras de esgoto no Brasil, provenientes de uma ETE. Neste estudo também foi demonstrada a eficiente remoção dos indicadores bacterianos, coliformes totais e fecais (99,9%) pelo tratamento da ETE com lodo ativado.

5.1.2 Rotavírus (Artigo 4.2)

Para a investigação de RVA, três protocolos de RT-PCR foram utilizados com objetivo de avaliar a eficiência de detecção. As técnicas amplificam diferentes genes de RVA (VP4, VP7 e VP6) (Gentsch et al. 1992; Das et al. 1994; Gouvea et al. 1994; Iturriza-Gomara et al. 2002). Os segmentos gênicos VP4 e VP7 codificam para as proteínas mais externas do capsídeo viral. Essas duas proteínas têm a função de reconhecimento e adesão ao receptor celular para a entrada do vírus para o interior da célula e são altamente imunogênicas. O segmento gênico VP6 codifica para a proteína mais abundante do capsídeo viral, que compõe o capsídeo interno dos rotavírus (Estes & Kapikian 2007).

A análise conjunta dos resultados obtidos em amostras de efluente e afluente demonstrou maior detecção de RVA pelo protocolo de VP6 [19/48(39,6%)] seguido pelo de VP4 [(17/47(36,2%)] e VP7 [14/47 (29,8%)].

Neste estudo tentou-se estabelecer uma metodologia quantitativa para RVA, entretanto os resultados não foram satisfatórios (dados não mostrados) e não se pôde determinar a eficiência do método de concentração utilizado. Considerando a importância epidemiológica dos RVA e a alta prevalência de infecções virais no Brasil a detecção de 39% (19/48) de amostras positivas para RVA em amostras de águas residuárias, pode ser considerado um valor baixo de detecção viral. Esse baixo percentual de detecção pode ser explicado pela baixa sensibilidade de detecção da técnica molecular ou pelo baixo percentual de recuperação do método considerando os resultados obtidos com HAstV (artigo 4.1) e NoV (artigo 4.3).

O protocolo de amplificação do gene VP6 permitiu a caracterização de mais um gene, como recomendado pela atual classificação de RVA (Matthijnssens et al. 2011). Entretanto, a utilização da amplificação baseada nos genes VP4 e VP7 têm a vantagem de classificar os genótipos de rotavírus em genótipos P (VP4) e G (VP7), comumente empregada na classificação binária e utilizada para se estabelecer a distribuição mundial dos genótipos. Estes protocolos de semi-nested PCR com iniciadores específicos para os genótipos mais comuns de RVA é capaz de genotipar os vírus detectados sem a necessidade de sequenciamento.

A caracterização dos genótipos P e G de RVA é de grande importância para o estudo e acompanhamento epidemiológico das infecções causadas pelos RVA. Em contrapartida, a sensibilidade é diminuída pela não detecção de genótipos menos usuais, possivelmente presentes em matrizes ambientais. Baseado nesse fato torna-se importante a utilização da RT-PCR baseada no segmento VP6, devido ao ganho de sensibilidade para investigação e também para caracterização deste gene para as amostras negativas ou positivas não-tipadas pelos genes VP4 e VP7.

A não obtenção de um método para quantificação de RVA neste primeiro estudo resultou em um trabalho posterior (artigo 4.4), para avaliação de um novo protocolo de quantificação pela amplificação do gene NSP3, assim como para avaliação da taxa de recuperação viral do método de filtração comparando como método de ultracentrifugação.

5.1.3 Norovírus (Artigo 4.3)

Para investigação de NoV três técnicas moleculares de detecção foram utilizadas: RT-PCR (Beuret et al. 2002), utilizado rotineiramente para o diagnóstico de NoV a partir de amostras clínicas; seminested RT-PCR (Boxman et al. 2006) utilizado para amostras ambientais e qPCR (Kageyama et al. 2003), sendo este último também utilizado para avaliação da taxa de recuperação de NoV.

NoV foi detectado em 7, 14 e 28 amostras de um total de 48 coletadas, utilizando PCR, seminested PCR e qPCR, respectivamente, demonstrando uma maior sensibilidade da técnica de qPCR para detecção destes vírus nas amostras analisadas. Porém, a utilização da PCR para detecção de NoV não se mostrou eficiente para estudos de detecção viral em amostras de água residuárias. Em estudo anterior, realizado em Manaus (Miagostovich et al. 2008), este mesmo protocolo revelou a baixa freqüência de contaminação por estes vírus. A escolha do método de detecção, como demonstrado por Ferreira e colaboradores (2009) pode influenciar os resultados das análises de agentes virais. Neste estudo o qPCR se mostrou mais

sensível para detecção de NoV, demonstrando que a utilização da técnica de PCR poderia afetar os resultados, subestimando a presença de NoV nas amostras estudadas. Outra grande vantagem da utilização do qPCR é a quantificação da carga viral nas amostras analisadas, tornando possível realizar estudos de sazonalidade, carga viral presente em águas residuárias e eficiência dos métodos de tratamentos utilizados pelas ETEs. Estudos têm demonstrado alta freqüência de detecção de NoV utilizando qPCR em amostras de água residuárias (Katayama et al. 2008; Kitajima et al. 2009).

No presente manuscrito, a quantificação dos NoV nas amostras coletadas durante o ano de estudo demonstrou um pico de carga viral entre os meses de junho e julho. Essa possível sazonalidade observada está de acordo com a maior circulação de NoV nos meses mais frios, descritos em outros estudos (Haramoto et al. 2006; Silva et al. 2007; Katayama et al. 2008).

A baixa eficiência de recuperação de NoV demonstrada pelo método de recuperação viral (Katayama et al. 2002) em águas residuárias neste estudo foi similar aos resultados obtidos para recuperação de HAstV (Guimarães et al. 2008). A eficiência recuperação em torno de 5%, para amostras de águas residuárias, demonstrou a necessidade de buscar novas metodologias para concentração viral nesta matriz.

Este foi o primeiro estudo realizado no Brasil demonstrando a presença de NoV em águas residuárias coletadas em uma ETE. O estudo destaca a resistência destes agentes virais após o tratamento e a importância da escolha do método de detecção viral.

5.1.4 Poliomavírus JC (Artigo 4.4)

O estudo da presença de JCPyV em amostras ambientais foi um estudo pioneiro no Brasil, sendo o primeiro estudo a determinar os genótipos de JCPyV circulantes no país. Neste estudo foi padronizado a metodologia de detecção e quantificação viral, o que permitiu avaliar a presença, a carga viral e estudar os genótipos circulantes nas amostras de afluente e efluente da ETE Fiocruz.

Os JCPyV foram detectados utilizando as metodologias de nested PCR (Bofill-Mas et al. 2000) e qPCR (Pal et al. 2006). As duas metodologias apresentaram resultados de detecção similares, demonstrando a possibilidade de utilização de ambas. Os JCPyV foram detectados em 100% das amostras de esgoto bruto corroborando sua alta excreção urinária e sua ampla distribuição em amostras de esgoto bruto. Diversos estudos descreveram a alta circulação de JCPyV em amostras de esgoto de outras regiões do mundo (Bofill-Mas et al. 2000; Albinana-Gimenez et al. 2006; Bofill-Mas et al. 2006; Katayama et al. 2008; Rafique & Jiang 2008).

Em amostras de esgoto tratado, JCPyV foram detectados em 43% (10/23) das amostras testadas.

A concentração de JCPyV nas amostras de esgoto bruto [média de 10^6 cópias de genoma por litro (CG/L)] foram similares as concentrações encontradas por Boffil-Mas e colaboradores (2006) em amostras de esgoto doméstico de Barcelona, Espanha. A redução da carga viral pelo tratamento utilizado na ETE Fiocruz foi observada pela redução do número de amostras positivas no efluente da ETE. A carga viral foi reduzida a níveis não detectáveis quando analisadas pelas metodologias (nested PCR e qPCR).

A utilização de duas metodologias sensíveis de detecção viral possibilitou a quantificação (qPCR) e a caracterização dos genótipos de JCPyV (nested PCR) detectados nas amostras analisadas. O seqüenciamento e posterior análise filogenética demonstrou que os genótipos de JCPyV deste estudo, agruparam com genótipos que circulam na África e na Europa. Existe uma hipótese da possível relação entre a migração humana pelos continentes e determinado genótipo circulante num dado grupo populacional (Stoner et al. 2000). A origem africana e européia dos vírus detectados no Brasil corrobora com a origem da população brasileira (Parra et al. 2003).

O presente estudo demonstrou a ampla distribuição e presença dos JCPyV em águas residuárias coletadas na ETE Fiocruz, Rio de Janeiro, fortalecendo a possibilidade da utilização destes vírus como marcadores de contaminação viral em águas ambientais.

5.1.5 Estabelecimento de metodologia de multiplex qPCR para detecção simultânea de RVA e do controle interno (bacteriófago PP7) e avaliação do método de ultracentrifugação (Artigo 4.5)

O baixo percentual de detecção de RVA em águas residuárias encontrados por Ferreira e colaboradores (2009) durante um ano de coletas quinzenais na ETE Fiocruz, associado à baixa taxa de recuperação de HAstV (4%) (Artigo 4.1) e NoV (5%) (Artigo 4.3) observada a partir de águas residuárias (ETE-Fiorcuz) apontou a necessidade de padronizar um novo método de concentração viral a partir desta matriz. Neste estudo, foi realizada a padronização do método de concentração viral, a partir de águas residuárias, baseado em ultracentrifugação (Pina et al. 1998). Para avaliação da eficiência de recuperação viral, o RVA foi utilizado como modelo experimental e para este propósito, a metodologia de qPCR para quantificação de RVA baseada na amplificação do gene NSP3 foi estabelecida. Este protocolo, previamente desenvolvido por Zeng e colaboradores (2008), por ser baseado na amplificação de um dos

segmentos gênicos mais conservados do genoma dos RVA, apresenta sensibilidade elevada de detecção e quantificação viral.

Um importante diferencial deste estudo foi a implementação da utilização de um CI para os métodos de concentração viral. Em estudos de virologia ambiental, a presença de inibidores naturais presentes nas amostras analisadas pode se tornar, muitas vezes, um obstáculo para detecção dos agentes virais, interferindo principalmente nas técnicas moleculares (Schwab et al. 1995; Ijzerman et al. 1997). Neste estudo, foi estabelecida a utilização do bacteriófago PP7 como CI (Rajal et al. 2007), inoculado anteriormente a etapa de concentração viral, e foi padronizado multiplex qPCR para detecção simultânea de RVA e PP7, baseado em iniciadores descritos por Zeng e colaboradores (2008) e Rajal e colaboradores (2008), respectivamente. A utilização de um CI é preconizada como uma importante ferramenta para os ensaios moleculares a fim de eliminar resultados falso-negativos (Hoorfar et al. 2004).

Para a padronização do multiplex qPCR, curvas padrão para RVA e bacteriófago PP7 foram geradas. Inicialmente foram padronizados as metodologias monoplex para cada vírus e em seguida, foi realizado o teste em formato multiplex. Os valores de Ct para cada ponto de diluição do DNA plasmidial utilizado na curva padrão foram comparados e não foi observada diferença comparando-se os ensaios mono e multiplex. As vantagens da utilização do qPCR incluem a alta sensibilidade para detecção viral, a obtenção de resultados quantitativos em menos tempo de execução e o menor risco de contaminação cruzada. Além disso, o formato multiplex desenvolvido durante este estudo diminui o custo final de análise.

Após a padronização do multiplex qPCR, amostras de águas residuárias foram coletadas e inoculadas com suspensão de RVA e PP7 para avaliação da eficiência de recuperação viral e para verificação da utilização do bacteriófago PP7 como CI. O método de concentração viral baseado em ultracentrifugação (47% - eficiência de recuperação) foi mais eficiente para a concentração de RVA quando comparado ao método previamente utilizado no LVCA (adsorção-eluição; 3,5% - eficiência de recuperação). O método descrito por Pina e colaboradores (1998) apresenta outra vantagem que é o custo reduzido para laboratórios que já possuam ultracentrifuga. Os reagentes e materiais necessários para realização da concentração viral por este método são de baixo custo, além dos tubos utilizados para a etapa de centrifugação poderem ser esterilizados e reutilizados.

A utilização do PP7 como CI foi bem sucedida, pois o mesmo pôde ser detectado em todas as amostras previamente inoculadas. Amostras ambientais, principalmente amostras de águas residuárias, podem conter grande quantidade de substâncias inibidoras, podendo inibir a detecção viral e resultar num falso negativo (Ijzerman et al. 1997). A utilização do CI em

amostras ambientais, da ultracentrifugação para concentração viral somado ao estabelecimento do multiplex qPCR agregou alto valor a este conjunto de técnicas por conter as características necessárias para uma boa metodologia de rotina aplicada em laboratórios de pesquisa: sensibilidade, reprodutibilidade, consistência e baixo custo.

Este estudo foi de grande importância para o estabelecimento de uma nova metodologia de concentração viral eficiente a partir de águas residuárias no LVCA e para a padronização da utilização de um CI para os futuros estudos em virologia ambiental. A utilização do PP7 como CI e sua detecção após todas as etapas laboratoriais é uma garantia, que, juntamente com todas as Boas Práticas Laboratoriais utilizadas no LVCA, oferece mais credibilidade e fidelidade aos resultados obtidos em nossas pesquisas.

5.2 Detecção e quantificação de vírus na ETE Alegria

Os estudos referentes aos artigos 4.6 e 4.7 foram realizados na ETE Alegria, dentro do acordo de cooperação técnica estabelecido entre a Fundação Oswaldo Cruz (nº 25380.003142/2009-79) e a Companhia Estadual de Águas e Esgotos-CEDAE (nº E – 17/ 100.905/2011) e encontram-se inseridos nas atividades da Fiocruz como Centro Colaborador PAHO/WHO em Saúde Pública e Ambiental.

A ETE Alegria recebe esgoto de aproximadamente 1,5 milhão de habitantes do centro e bairros da zona norte do Rio de Janeiro, e é uma das maiores ETE do Brasil. É uma planta de tratamento secundário que aplica o tratamento aeróbico de lodo ativado sem posterior cloração do efluente. Ao final de todo o tratamento, o efluente é descartado na Baía de Guanabara, perfazendo seu papel no programa de despoluição da Baía de Guanabara.

Nos estudos a seguir, foram analisados os vírus DNA, que atualmente estão apontados como possíveis marcadores de contaminação viral, (HAdV e JCPyV) e os vírus RNA, principais agentes causadores de GA infantil (RVA, NoV e HAstV).

5.2.1 Avaliação da disseminação de RVA em águas residuárias e estudo dos genótipos circulantes na população do Rio de Janeiro, RJ, após a introdução da vacina anti-RVA (Rotarix®) no Brasil (Artigo 4.6)

Após a avaliação de um novo método de concentração viral e a padronização da utilização do PP7 como CI e sua detecção, em conjunto com RVA, por multiplex qPCR, foi desenvolvido um estudo epidemiológico da prevalência e distribuição de RVA em uma ETE de grande vazão. Este tipo de abordagem tem sido utilizado em diversos estudos para obter

informações a respeito da circulação viral na população atendida pela ETE, independente de casos não reportados e infecções assintomáticas (Gajardo et al., 1995; Bosch et al., 2008; Haramoto et al., 2008; Clemente-Casares et al., 2009).

No artigo "One year environmental surveillance of rotavirus specie A (RVA) genotypes in circulation after the introduction of the Rotarix® vaccine in Rio de Janeiro, Brazil" foi demonstrada a alta prevalência e a alta carga viral em amostras de águas residuárias coletadas na ETE Alegria. O RVA foi detectado por qPCR em todos os meses do ano em ambas as amostras, não apresentando a sazonalidade nos meses mais frios, como previamente descrita para esses agentes (Kane et al. 2004; Carvalho-Costa et al. 2011).

Neste estudo, após a reação de transcrição do genoma viral, foram empregadas diferentes variantes da PCR (qPCR e nested-PCR) para detecção, caracterização e quantificação dos RVA nas amostras de água residuárias. Cada uma das técnicas apresenta um determinado segmento gênico como alvo de amplificação. A metodologia de qPCR, descrita por Zeng e colaboradores (2008), se mostrou a mais sensível para detecção, com a vantagem adicional de quantificação da carga viral nas amostras testadas. Com a utilização desta metodologia foi demonstrado uma redução de aproximadamente dois logs na carga viral de RVA comparando amostras de afluente e efluente a ETE, enfatizando o papel das plantas de tratamento de esgoto na redução da carga de contaminantes microbiológicos lançados no meio ambiente.

Após a introdução da vacina anti-RVA (Rotarix®, GlaxoSmithKline, Rixensart, Bélgica) no calendário de vacinação pelo PNI do Brasil em março de 2006, a vigilância dos genótipos de RVA circulantes na população brasileira se tornou extremamente relevante.

A vigilância ambiental, utilizando amostras de ETE, pode ser uma alternativa para o Brasil e outros países em desenvolvimento que esbarram no alto custo e em outras dificuldades existentes para a execução de um programa ativo de vigilância utilizando amostras clínicas, para caracterizar os genótipos de RVA circulantes em uma determinada população. No presente estudo, os genótipos G2, P[4] e P[6] foram os mais prevalentes durante o estudo. Esses resultados condizem com a circulação dos genótipos analisados pela vigilância em amostras clínicas (Carvalho-Costa et al. 2009; 2011) e dados provenientes do LVCA, referentes à genotipagem de amostras clínicas positivas para RVA, no mesmo período do presente estudo e corrobora a prevalência do genótipo G2P[4] na população da cidade do Rio de Janeiro. O presente estudo, analisando amostras ambientais, demonstra um cenário de predominância dos genótipos de RVA G2 e P[4] relatado por estudos clínicos (Carvalho-Costa et al. 2009; 2011). A alta prevalência deste genótipo tem sido observada tanto em países que introduziram a vacina monovalente G1P[8] (Rotarix®), como em países onde não houve

influência da vacinação (Ferrera et al. 2007; Antunes et al. 2009; Carvalho-Costa et al. 2009; 2011). Esta mudança no perfil de prevalência de genótipos pode ser explicada por uma flutuação genotípica natural dos RVA, contudo o papel exercido pela introdução da vacina anti-RVA não pode ser descartado (Gómez et al. 2011).

Neste estudo, também foi realizado a busca por vírus vacinais no meio ambiente nas amostras de água residuárias. Duas abordagens foram aplicadas para a identificação de RVA de origem vacinal, baseadas na amplificação de dois segmentos gênicos: NSP3 e NSP4. Esses dois segmentos foram seqüenciados no LVCA e, comparando com segmentos de RVA selvagem, foi possível identificar mutações nucleotídicas que posteriormente foram utilizadas para diferenciação entre RVA selvagem e vacinal. Inicialmente, o gene NSP4 foi amplificado e seqüenciado para análise das mutações relativas à vacina. Não foram encontradas as mutações nas sequências analisadas. Um estudo filogenético classificou o segmento NSP4 como pertencentes a E1 (1/10) e E2 (9/10). Nenhuma das 10 amostras apresentou as mutações relativas ao gene vacinal. A maior predominância de genótipos E2 está em concordância com a alta prevalência de genótipos G2 e P[4] encontrados, segundo o agrupamento usual de genótipos de RVA descrito por Matthijnssens e colaboradores (2011). Baseado na amplificação do gene NSP3 e na metodologia descrita por Rose e colaboradores (2010), cinco amostras de afluente foram clonadas e destas, 44 colônias foram analisadas para a verificação do perfil eletroforético após a reação de digestão. Não foi observada nenhuma colônia contendo o segmento gênico NSP3 derivado da vacina. A ausência ou a baixa carga de RVA de origem vacinal no meio ambiente, demonstrado por resultados negativos, pode ser explicada pela manutenção de altas taxas de infecções naturais causadas pelos RVA e no fato de que a Rotarix® é disponibilizada continuamente nos postos de saúde para aplicação.

5.2.2 Avaliação de marcador virológico de contaminação humana (Artigo 4.7)

No manuscrito “Assessment of viral agents’ burden in an urban sewage treatment plant located in Rio de Janeiro, Brazil” os vírus DNA (HAdV e JCPyV) e os vírus RNA (RVA, NoV e HAstV) foram detectados e quantificados de afluentes e efluentes coletados durante um ano de estudo na ETE Alegria. O principal objetivo deste estudo foi demonstrar a carga presente nas amostras de água residuárias, com a finalidade de gerar dados a respeito da presença e concentração de agentes virais em amostras provenientes de um país em desenvolvimento. A necessidade de um marcador para indicar a presença viral em amostras ambientais é um consenso entre virologistas, e a indicação de alguns agentes, como os HAdV e dos JCPyV, é baseada principalmente em estudos realizados de amostras coletadas em

países desenvolvidos (Puig et al. 1994; Pina et al. 1998; Laverick et al. 2004; Bofill-Mas et al. 2006; Fong et al. 2010; Haramoto et al. 2010).

Neste estudo, o qPCR foi utilizado para quantificar os agentes virais e demonstrou a maior prevalência dos vírus DNA e dos RVA nas amostras de águas residuárias. Nas amostras de afluente, 100% (24/24) foram positivas para HAdV, JCPyV e RVA, enquanto que NoV e HAstV foram detectados em 55% e 29%, respectivamente. Nas amostras de efluente, contudo, os JCPyV (17%) foram detectados em menor proporção comparado aos HAdV (75%) e RVA (71%).

A contribuição deste estudo com dados quantitativos e de prevalência dos principais vírus entéricos presentes em águas ambientais é de grande relevância para a determinação de marcadores virais de contaminação fecal de origem humana. Estes dados são originais e pioneiros em relação à presença e quantificação viral destes agentes em país em desenvolvimento. Os resultados corroboraram com outros estudos (Bofill-Mas et al. 2006; Fong et al. 2010; Haramoto et al. 2010) a respeito da alta prevalência e carga viral dos HAdV em águas residuárias, contudo demonstrou diferente padrão de circulação viral, principalmente em relação a alta detecção e concentração de RVA nas amostras coletadas nesta ETE. Ressalta-se a importância epidemiológica que os RVA possuem no Brasil como um dos principais agente causadores de GA infantil, como demonstrado anteriormente (Leite et al. 2008; Carvalho-Costa et al. 2009; 2011). A alta detecção de HAdV no Brasil, corroborando com dados de diferentes regiões do mundo, reforça a possibilidade de utilização destes agentes como marcadores virais de contaminação fecal de origem humana. Contudo, a alta detecção dos RVA tanto em amostras de afluentes como efluentes, chama a atenção para as diferenças na distribuição epidemiológica viral para diferentes regiões do mundo, que deve ser levado em consideração ao se estabelecer padrões universais.

A caracterização dos genótipos virais de HAdV, JCPyV e NoV neste estudo refletiu dados epidemiológicos baseados na vigilância em amostras clínicas. Este fato demonstra a utilização de amostras de esgoto bruto para fins de caracterização dos genótipos virais circulantes na população local. Como a ETE Alegria atende uma população representativa da cidade do Rio de Janeiro é possível realizar estudos mais detalhados de genotipagem, como a clonagem e sequenciamento das amostras positivas, e estabelecer uma vigilância epidemiológica ambiental para a identificação dos genótipos virais circulantes na cidade durante um determinado período de estudo.

Os dados obtidos neste estudo demonstram a diminuição da carga viral após o tratamento de esgoto e reforça a importância ao tratamento de esgoto doméstico antes do descarte no meio ambiente. Com o crescimento populacional e a concentração de grandes

aglomerados humanos em centros urbanos, o investimento na construção de ETEs é uma saída para diminuir o impacto humano à natureza, contribuindo assim para a melhoria da saúde da população.

6. CONCLUSÕES

- O monitoramento viral da ETE Fiocruz na cidade do Rio de Janeiro demonstrou a circulação de HAstV, NoV GI e GII, RVA e pela primeira vez a caracterização de genótipos de JCPyV no país em amostras de água residuárias.

- O baixo percentual de recuperação de HAstV somado, a baixa prevalência destes agentes em casos de GA refletem na menor freqüência de detecção destes vírus em amostras de águas residuárias.

- Diferentes percentuais de detecção de NoV demonstraram a importância de se considerar diferentes metodologias de detecção, porém ficou demonstrado a circulação predominante de NoV GII com pico de detecção nos meses de inverno.

- Os genótipos G1 e P[8] de RVA foram os mais prevalentes no estudo realizado em 2004-2005, anterior a introdução da vacina anti-rotavírus, porém a caracterização dos genótipos G2 e P[4] em quase 100% das amostras detectadas (2009-2010), após a introdução da vacina, revelaram a mudança dos genótipos circulantes no país.

- O método de ultracentrifugação se mostrou mais eficiente para recuperação de RVA em amostras de águas residuárias, e a detecção molecular destes vírus por PCR demonstrou a maior sensibilidade baseada na amplificação do segmento VP6, comparado a amplificação dos segmentos VP4 e VP7.

- O estabelecimento do multiplex qPCR para detecção de RVA e do bacteriófago PP7 apresentou sensibilidade similar aos resultados obtidos com o monoplex qPCR, e a utilização do bacteriófago PP7 como controle interno forneceu confiabilidade aos resultados obtidos.

- Não foi possível demonstrar a circulação de cepas vacinais de RVA nas amostras ambientais durante o período de estudo.

- O monitoramento da ETE Alegria demonstrou a maior prevalência de RVA, HAdV e JCPyV com concentrações superiores a 10^5 CG/L em esgoto bruto e uma menor circulação de NoV e HAstV, com percentuais de detecção de 31% e 17%, respectivamente.

- A caracterização dos genótipos de JCPyV corroborou com dados obtidos anteriormente em amostras clínicas e ambientais, onde foi observado a maior circulação dos genótipos de origem africana (AF2) no Rio de Janeiro.

- A quantificação viral nesta ETE demonstrou a alta de concentração de HAdV em amostras de esgoto tratado, reforçando o potencial destes vírus como marcadores de contaminação ambiental.

- Os resultados obtidos demonstraram uma redução da carga viral após o tratamento esgoto realizado pelas ETEs, contudo a presença de vírus em amostras de esgoto tratado alerta a respeito da disseminação viral para o meio ambiente a partir de efluentes tratados.

7. PERSPECTIVAS

O despejo de esgotos tratados e não tratados constituem a principal fonte de contaminação viral para o meio ambiente. Nesta tese foi demonstrada a circulação em altas concentrações de diferentes agentes virais com potencial patogênico para o homem.

O risco de infecção viral via água contaminada está diretamente relacionado ao contato do homem com esta matriz. Neste aspecto águas costeiras utilizadas para recreação, como praias, devem ser avaliadas são os principais meios de contato entre agente viral e hospedeiro.

Sendo o Rio de Janeiro uma cidade litorânea e turística, a perspectiva deste trabalho é estabelecer no LVCA uma metodologia eficiente de concentração viral a partir de água do mar, realizando um estudo piloto em uma praia do Rio de Janeiro. Como planos futuros, teremos como objetivos: 1) Determinar a contaminação dos vírus investigados nesta tese em águas costeiras da cidade; e 2) Estabelecer metodologias para realização de testes de viabilidade viral nas amostras positivas detectadas.

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