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APLICAÇÃO DE MÉTODOS MOLECULARES E DE CULTIVO CELULAR NO MONITORAMENTO DE VÍRUS ENTÉRICOS NO AMBIENTE AQUÁTICO

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“O homem pode tanto quanto sabe”
Francis Bacon

RESUMO

Os vírus entéricos humanos são importantes causas de enfermidades veiculadas através da água. Atualmente, em termos de legislação brasileira, apenas a contagem do número de coliformes é utilizada para determinar a segurança microbiológica de águas tratadas e não tratadas. Os vírus presentes no meio ambiente são, na sua maioria, não adaptados ao cultivo i*n vitro*, tornando-se necessário o desenvolvimento e implementação de métodos que possibilitem a adoção de medidas preventivas para controle de contaminação viral. Assim sendo, os objetivos do presente trabalho foram (I) padronizar e estabelecer metodologias de concentração, detecção, quantificação e viabilidade viral no ambiente aquático; (II) realizar a pesquisa de adenovírus humanos (HAdV), norovírus (NoV) genogrupos GI e GII, rotavírus humanos genogrupo A (RV-A) e vírus da hepatite A (HAV) em águas ambientais e ostras de cultivo durante o período de um ano e (III) avaliar por ensaio de placa de lise a sobrevivência de HAdV infecciosos sorotipos 2 e 41 em águas de superfície e subterrâneas. No estudo de padronização dos métodos foram utilizados como modelos os HAdV e HAV inoculados em águas: destilada, de esgoto tratado, do mar e da lagoa. A melhor eficiência de recuperação viral (100%) foi obtida quando as matrizes de água destilada e de esgoto tratado foram utilizadas. A menor eficiência (10%) foi encontrada para a água do mar. No estudo de campo, águas foram coletadas de 8 locais de Florianópolis, Santa Catarina e ostras (*Crassostrea gigas*) de duas fazendas de cultivo (norte e sul da Ilha) foram também avaliadas no mesmo período. Os resultados de detecção de genomas virais foram HAdV: 75% (esgoto tratado); 64,2% (águas ambientais); 87,5% (ostras); RV-A: 41,6% (esgoto tratado); 19% (águas ambientais); 8,3% (ostras); HAV: 25% (esgoto tratado); 8,3% (águas ambientais); ausência nas ostras; NoV: 50% (esgoto tratado); 19% (águas ambientais); ausência nas ostras. No estudo de viabilidade viral por PCR integrado a cultura celular (ICC-PCR), confirmou-se positividade de HAdV em: 66,6% (esgoto tratado); 83,8% (águas ambientais); de RV-A: ausência em esgoto tratado e 12,5% (águas ambientais); de HAV: 66,6% (esgoto tratado) e ausência nas águas ambientais. Por PCR quantitativo o número de genomas (gc) de NoV nas águas foram médias de $1,8 \times 10^2$ (GI) e $1,0 \times 10^3$ gc/L (GII) em esgoto tratado e de $1,1 \times 10^2$ (GI) e $8,1 \times 10^3$ gc/L (GII) nas águas ambientais. Para HAdV obteve-se médias de: $9,8 \times 10^4$ (esgoto tratado); $9,8 \times 10^6$ gc/L (águas ambientais) e de $9,1 \times 10^4$ (ostras sul da ilha) e $1,5 \times 10^5$ gc/g (ostras norte da ilha). Os resultados obtidos indicam uma maior prevalência de HAdV no ambiente aquático, seguido de NoV, RV-A e HAV. No estudo de decaimento de infectividade de HAdV em amostras de água houve uma significativa inativação viral somente ao final das 23 semanas a 19°C para os dois sorotipos de adenovírus testados. Estes resultados demonstram a longa taxa de sobrevivência destes vírus em águas ambientais.

Palavras-chave: Reação em cadeia de polimerase, Água – Qualidade, Vírus entéricos, Cultivo celular.

ABSTRACT

Enteric viruses are important cause of gastroenteritis outbreaks transmitted through contaminated water sources. Nowadays, the Brazilian legislation only requires the coliforms counting to determine the microbiologic safety in treated and non treated water. Most viruses present in the aquatic environment are not adaptable to *in vitro* cultures, becoming necessary to develop and implement methods that could be used in the monitoring and prevention of viral contamination. Therefore, the objectives of this thesis were (I) to standardize and establish methodologies of concentration, detection, quantification and viral viability in the aquatic environment; (II) to access the contamination of human adenovirus (HAdV), norovirus (NoV) genogrups GI e GII, human rotavirus genogrup A (RV-A) and Hepatitis Virus A (HAV) in environmental waters and oysters during one year; (III) to study the survival of infectious HAdV type 2 and 41 in surface and ground waters measured by plaque assay. In the standardization study, HAdV and HAV were used as models and were inoculated in distilled water, treated wastewater, seawater and lagoon water, showing better virus recovery (100%) in distilled water and treated wastewater, and lower recovery (10%) in seawater. In the field study, water samples were collected from 8 sites in Florianopolis, Santa Catarina and oyster samples (*Crassostrea gigas*) from two oysters' farms (north and south of the Island) were also collected in the same period. The detection results of virus genomes were HAdV: 75% (treated wastewater); 64.2% (environmental waters); 87.5% (oysters); RV-A: 41.6% (treated wastewater); 19% (environmental waters); 8.3% (oysters); HAV: 25% (treated wastewater); 8.3% (environmental waters); absence in oysters; NoV: 50% (treated wastewater); 19% (environmental waters); absence in oysters. In the viability study by integrated cell culture PCR (ICC-PCR), the results confirmed viable HAdV: 66.6% (treated wastewater); 83.3% (environmental waters); RV-A: absence in treated wastewater and 12.5% (environmental waters); HAV: 66.6% (treated wastewater) and absence in environmental waters. By quantitative PCR assays the number of genomes (g.c.) for NoV in waters were averages of 1.8 x 10^2 (GI) and 1.0 x 10^3 gc/L (GII) in treated wastewater and 1.1 x 10^2 (GI) and 8.1 x 10^3 gc/L (GII) in environmental waters. For HAdV the averages were: 9.8 x 10^4 (treated wastewater); 9.8 x 10^6 gc/L (environmental waters) and 9.1 x 10^4 (oyster farm South) e 1.5 x 10^5 gc/g (oyster farm North). The surveillance results have shown a higher prevalence of HAdV in the aquatic environment, followed by NoV, RV-A and HAV. In the study of HAdV infectivity decay in water under different temperatures, results showed a significant inactivation only after 23 weeks at 19°C for both HAdV tested. These results have shown a long-term survival of adenoviruses in environmental waters.

Keywords: Polymerase Chain Reaction, Water quality, Enteric viruses, Cell Culture.

SUMÁRIO

1. INTRODUÇÃO

1.1. Aspectos Gerais

A pesquisa de vírus na água iniciou-se após a ocorrência de um surto de hepatite em Nova Délhi (Índia), na década de 1950, como conseqüência da contaminação do sistema de tratamento da água por patógenos virais provenientes do esgoto não tratado. Após esse episódio, iniciaram-se os estudos na área da virologia aquática, atualmente denominada virologia ambiental, com cientistas tentando detectar poliovírus em amostras de água (BOSCH, 1998; TAVARES et al., 2005).

Desde 1990, com a implementação de métodos moleculares no estudo de vírus em amostras ambientais, vem crescendo a atenção dada à contaminação da água, do solo e dos alimentos pelos vírus. A pesquisa de vírus em águas tratadas e não tratadas hoje em dia tornou-se uma relevante linha de investigação em desenvolvimento no mundo, visando à garantia da qualidade da água usada pela população.

Os vírus são considerados a principal causa de doenças transmitidas pela água e têm sido responsáveis por 30 a 90% das gastroenterites no mundo (BOSCH et al., 2008). Dentre o diverso grupo dos vírus, encontram-se os vírus entéricos. O termo “vírus entérico” compreende todos aqueles que estão presentes no trato gastrintestinal humano e podem ser eliminados nas fezes durante o período de replicação. Eles são representados por diversas famílias comumente estudadas: Picornaviridae (poliovírus, enterovírus, coxsakievírus, vírus da hepatite A, e echovírus), Adenoviridae (adenovírus), Caliciviridae (norovírus e saporovirus), Astroviridae (astrovírus) e Reoviridae (rotavírus).

Os vírus entéricos se encontram distribuídos em todas as partes do mundo, estando presentes nas águas de esgoto que, ao serem lançadas em rios, córregos e até no mar, apresentam um grande risco à população, pois são responsáveis por diversas doenças que afetam o ser humano, tais como gastroenterites, meningites, miocardites e hepatites infecciosas (PINA et al., 1998).

De acordo com a Organização Mundial da Saúde (OMS), 1,1 bilhão da população mundial consomem água não tratada (KINDHAUSER, 2003). Aproximadamente 1,5 bilhão de casos de diarréia principalmente em crianças abaixo de cinco anos, ocorrem anualmente nestes países, em virtude das condições precárias de saneamento básico, higiene e tratamento da água (ASHBOLT, 2004).

No Brasil, apesar das melhorias nas condições de vida da população, ainda é baixo o número de domicílios com crianças de até 6 anos de idade que possuem condições adequadas de saneamento. Em 2007, apenas 54,5% dos domicílios com crianças possuíam todos os serviços de saneamento simultaneamente (IBGE, 2008). Este dado é relevante, tendo em vista que boa parte dos óbitos infantis tem causas ligadas à falta de saneamento básico, como, por exemplo, a diarréia. Portanto, a melhora do nível de saneamento tem impacto direto sobre as taxas de mortalidade infantil.

Surtos de doenças virais entéricas por veiculação hídrica têm sido descritos em muitos países e a lista de microorganismos potencialmente patogênicos aumenta anualmente (STRAUB; CHANDLER, 2003). Posteriormente à ingestão pelo homem, os vírus entéricos humanos se replicam no trato gastrointestinal e são eliminados em grandes quantidades juntamente com as fezes (10^8 a 10^{11} partículas virais/g de fezes). No meio ambiente, estes vírus são capazes de se agregarem rapidamente às partículas sólidas, protegendo-se desta forma dos fatores inativantes, o que favorece sua sobrevivência. Estes vírus possuem certa resistência aos agentes físico-químicos de inativação, podem permanecer viáveis ou potencialmente infectantes durante meses na água, resistindo a condições ambientais adversas e a processos de tratamento de água e esgoto, aplicados no controle bacteriano (LECLERC; SCHWARTZBROD; DEI-CAS, 2002; SKRABER et al., 2004; TREE; ADAMS; LEES, 2003).

O potencial turístico do Estado de Santa Catarina alcança expressão nacional, sendo que o município de Florianópolis encontra no turismo uma das principais fontes de renda de seus habitantes. A população flutuante, que praticamente triplica, durante a alta temporada de verão traz benefícios econômicos à população local, mas também aumenta a quantidade de esgoto doméstico produzido e lançado direta ou indiretamente nos ecossistemas aquáticos.

No Sul do Brasil, o Estado de Santa Catarina encontra-se entre os cinco Estados brasileiros mais afetados por poluição das águas, fornecendo condições ótimas para uma transmissão eficiente de vírus e parasitas e eventuais surtos de gastroenterites agudas (IBGE, 2008). Este fato entra em conflito com outra atividade de importância reconhecida na região, a maricultura. A qualidade microbiológica dos moluscos bivalves está intimamente relacionada com a qualidade sanitária do ambiente onde são cultivados, pois estes organismos são filtradores de água e podem acumular nos seus tecidos os patógenos presentes no ambiente. Os níveis de vírus presentes nos moluscos podem ser 100-1000 maiores do que suas concentrações nas águas onde são cultivados (CARTER, 2005).

Nos últimos anos, foi detectado um aumento da poluição nos locais de cultivo de moluscos no Estado de Santa Catarina, com a detecção da presença de patógenos virais e bacterianos nos moluscos e nas águas oceânicas (COELHO et al., 2003; RIGOTTO et al., 2005, SINCERO et al., 2006). Tendo em vista o significado da contaminação viral e bacteriana das águas, e por ser a maricultura uma atividade em crescimento e de grande importância econômica e social, o controle da qualidade sanitária dos produtos da maricultura se faz necessário e urgente (CRAUN, 1986; KOPECKA et al., 1993; PINA et al., 1998; SCHWAB et al., 2001).

Em geral, águas de recreação não sofrem nenhum tratamento sanitário, podendo conter altas concentrações virais, e ainda assim consideradas como próprias pela resolução do Conselho Nacional do Meio Ambiente (CONAMA-20, 20/12/2000) (BRASIL, 2000). Assim sendo, métodos para determinação de vírus entéricos em amostras de águas são necessários para monitorar e assegurar um padrão ideal de qualidade no suprimento de águas públicas.

Tradicionalmente, indicadores bacterianos como os coliformes fecais têm sido utilizados como indicadores de qualidade microbiológica da água, entretanto já está estabelecido mundialmente que os padrões bacterianos aceitos nem sempre predizem a presença de vírus nas águas ambientais (FORMIGA-CRUZ et al., 2002; PUSCH et al., 2005).

No Brasil, a Portaria 518 da ANVISA/MS, de 25/03/2004 recomenda que em complementação às análises de coliformes totais, termotolerantes e cianobactérias, ocorra a inclusão da pesquisa de organismos patogênicos, com o objetivo de atingir, como meta, um padrão de ausência, dentre outros, de enterovírus, cistos de *Giardia* spp. e oocistos de *Cryptosporidium* sp.. Porém, desde a publicação desta Portaria, ainda não houve mudança na lei brasileira no que se refere à obrigatoriedade de utilização destes microrganismos como padrão de potabilidade (BRASIL, 2004).

A rápida detecção e a caracterização molecular de vírus entéricos em água contribuem em ações epidemiológicas efetivas, abrindo novas perspectivas de controle ambiental em águas tratadas para consumo e irrigação, águas não tratadas, água mineral para consumo, estações balneárias, centros de maricultura que visam à produção de bivalves para consumo interno e/ou exportação, e na vigilância epidemiológica para caracterização dos genótipos circulantes e avaliação do impacto da introdução de futuras vacinas no ambiente (LINHARES, 2002; STRAUB; CHANDLER, 2003).

As normas para a avaliação da qualidade sanitária dos frutos do mar cultivados para consumo são asseguradas por métodos que dispensam a avaliação de coliformes fecais para moluscos, exigindo a análise de presença ou ausência de *Salmonella* sp. e *Streptococus*

coagulase positivo. Os níveis de coliformes fecais continuam exigidos apenas para as águas de cultivo (BRASIL, 2000).

Dentre os vírus que podem estar presentes no esgoto doméstico e, consequentemente no ambiente, destacam-se adenovírus, rotavírus, norovírus e vírus da hepatite A, que serão utilizados neste trabalho como modelos para a verificação da contaminação por vírus entéricos no ambiente aquático.

1.2. Adenovírus

Adenovírus (AdV) estão classificados na família Adenoviridae, que compreende quatro gêneros: Mastadenovírus, que infectam mamíferos (homens, símios, bovinos, equinos, caninos, suínos, ovinos e roedores); Aviadenovírus que infectam aves; Atadenovírus que infectam cervos, cobras e patos e Siadenovírus que infectam anfíbios e perus. Esses vírus apresentam uma grande variabilidade genética, o que possibilitou sua classificação em espécies e sorotipos (LEES, 2000).

O gênero Mastadenovirus é formado por mais de 90 sorotipos, entre os quais 52 deles infectam humanos [adenovírus humanos (HAdV)], divididos em seis espécies, A – F (JONES; DRANE; GOWANS, 2007). Os determinantes antigênicos importantes para a caracterização sorológica estão localizados no *hexon* e na fibra (Figura 1). Na superfície interna do *hexon* situa-se o determinante *alfa*, que é o antígeno Gênero-específico comum a todos os membros do mesmo Gênero. O determinante *epsilon* do *hexon* e o *gama* da fibra caracterizam os sorotipos (FLINT et al., 2000).

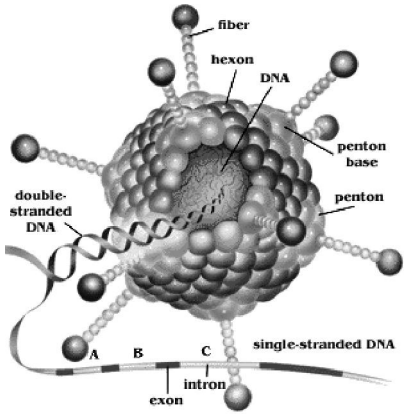

Figura 1: Representação esquemática da morfologia e estrutura dos adenovírus (www.nobel.se/medicine/education/poster/1993/genes-in-pieces.html).

Atualmente, HAdV são reconhecidos como agentes etiológicos causadores de diversas doenças, tais como infecções gastrointestinais, urinárias, do trato respiratório e do globo ocular (LEWIS et al., 2009). As infecções pelos HAdV são cosmopolitas e espécie-específicas, sendo a propagação feita de homem para homem; porém, por serem vírus estáveis podem ser transmitidos pela água (de consumo, em balneários ou piscinas) (MUNIAIN-MUJIKA et al., 2003; PAPAPETROPOULOU; VANTARKIS, 1998). Com poucas exceções, os sorotipos humanos não são patogênicos para os animais e os sorotipos de animais somente são patogênicos para as espécies de origem (HORWITZ, 1996).

Os membros do subgênero A (sorotipos 12, 18 e 31) causam infecções respiratórias e gastroenterites em crianças menores de um ano de idade. O subgênero B está dividido em dois grupos, pela análise de restrição do DNA. Na classe B1 encontram-se os sorotipos 3, 7, 14, 16 e 21, que são associados predominantemente às infecções respiratórias; na classe B2 encontram-se os sorotipos 11, 34 e 35, sendo bastante próximos geneticamente e causam infecções renais e do trato urinário (WADEL, 1984). Pesquisas recentes detectaram nos EUA um sorotipo variante do Ad14, o Ad14a, mais virulento e associado a casos graves de infecções respiratórias levando pacientes a óbito, e ilustram a plasticidade biológica destes vírus, sugerindo que outros adenovírus, incluindo os sorotipos entéricos, poderiam se tornar uma fonte de preocupação de saúde pública no futuro (GRAY; CHORAZY, 2009; LEWIS et al., 2009).

Os HAdV da espécie F, representada pelos HAdV 40 e HAdV 41, também denominados de adenovírus entéricos por causarem diarréias graves, principalmente em crianças, estão entre os agentes virais causadores dessa enfermidade (HORWITZ, 1996). Além desses, outros sorotipos de adenovírus estão relacionados com casos de gastrenterites infantis agudas, dentre os quais os HAdV 12, HAdV 18 e HAdV 31 (espécies A); HAdV 3 e HAdV 7 (espécies B); HAdV 1, HAdV 2 e HAdV 5 (espécies C) (BROWN, 1990; GOMES et al., 1989).

Vários estudos realizados em outros países, indicaram que os HAdV são importantes patógenos responsáveis por gastrenterites infantis, apresentando percentuais que variaram de 2,5 a 15%, sendo os HAdV 40 e HAdV 41, espécies F, prevalentes em, aproximadamente, 50% das amostras positivas (OH et al., 2003). No Brasil, estudos envolvendo diversos Estados demonstraram o envolvimento dos HAdV em casos de gastrenterites infantis agudas (ANDREASI et al., 2008; HÁRSI et al., 1995; PEREIRA et al., 1993; SOARES et al., 2002).

Em geral, a gastroenterite associada aos adenovírus entéricos é tão prevalente quanto a causada por rotavírus, e ocorre mais frequentemente em crianças com menos de quatro anos,

caracterizando-se como uma doença branda com diarréia e vômito. Em regiões de clima temperado, a prevalência de adenovírus entéricos é maior. No entanto, em países de clima tropical, como o Brasil, adenovírus veiculados pela água foram detectados durante todos os meses do ano (HORWITZ, 1996).

Sabe-se que os adenovírus apresentam maior estabilidade na água do que os enterovírus e outros vírus entéricos, porém poucos surtos de gastrenterites causados por adenovírus associados a águas destinadas ao consumo humano contaminadas têm sido evidenciados (FROST et al., 2002; LEE; KIM, 2002).

Geralmente, os sistemas de tratamento de água no Brasil e na maior parte do mundo utilizam cloro livre para desinfecção de águas de consumo, sendo eficazes na inativação de inúmeros microrganismos, inclusive os adenovírus. Entretanto, muitos sistemas de tratamento têm alternativamente usado a radiação ultravioleta (UV) como desinfecção primária, seguida do uso de monocloramina como desinfetante secundário (SIRIKANCHANA et al., 2008b). Esse método alternativo tem se mostrado efetivo na inativação de oocistos de *Cryptosporidium parvum*, que são muito resistentes ao método tradicional de tratamento com cloro livre, entretanto os adenovírus em particular, são mais resistentes à radiação UV do que qualquer outro vírus, bactéria, e protozoários presentes em água de consumo (BALLESTER et al., 2004; GERBA; PEPPER; WHITEHEAD, 2002; LINDEN et al., 2007; SIRIKANCHANA et al., 2008b; YATES; MALLEY; HOFFMAN, 2006). Além disso, os adenovírus são também resistentes à inativação com monocloramina, sugerindo que o sistema alternativo de tratamento com UV-monocloramina poderia não ser efetivo na remoção destes vírus (SIRIKANCHANA et al., 2008a).

1.3. Rotavírus

Os rotavírus (RV) pertencem à Família Reoviridae, gênero *Rotavirus*. A partícula viral completa mede aproximadamente 70-90 nm de diâmetro e é constituída por três camadas protéicas e um genoma com 11 segmentos de RNA fita dupla (dsRNA). Das proteínas codificadas por esses segmentos, seis são estruturais: VP1, VP2, VP3, VP4, VP6 e VP7, e cinco são não estruturais: NSP1, NSP2, NSP3, NSP4 e NSP5 (Figura 2). A proteína não estrutural NSP4 é uma glicoproteína codificada pelo segmento 10 e foi caracterizada como sendo uma enterotoxina viral capaz de induzir diarréia em camundongos jovens (KAPIKIAN; CHANOCK, 1996).

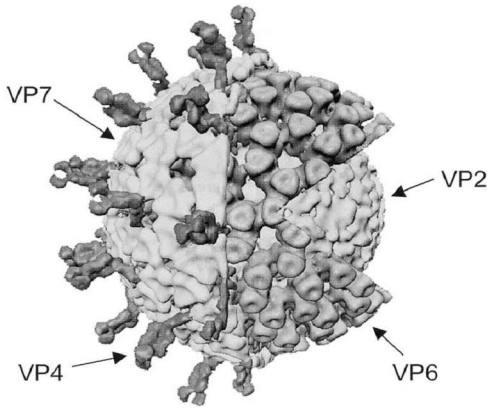

Figura 2: Representação de uma partícula de rotavírus, com suas respectivas proteínas: VP4, VP7, VP6 e VP2 (ARIAS et al., 2001).

Os RV são classificados em sete sorogrupos (A a G), reconhecidos com base na especificidade antigênica da proteína VP6. A maioria das amostras de origem humana pertence ao grupo A, embora ocasionalmente os grupos B e C estejam associados com doenças em humanos e os grupos D a G foram somente descritos em animais (ESTES, 1996). O grupo A é classificado em sorotipos e/ou genótipos. Os sorotipos G ou P são determinados pela reatividade do vírus em ensaios de neutralização com base nas proteínas VP7 ou VP4 do capsídeo, respectivamente. Os genótipos P ou G são determinados por sequenciamento, amplificação ou hibridização dos genes 4 ou 9, que codificam as proteínas VP4 ou VP7 (KAPIKIAN; CHANOCK, 1996).

A proteína estrutural VP6 é codificada por um gene muito conservado dentre todos os sorotipos/genótipos de rotavírus. Como ela pode ser comum à grande maioria dos sorotipos de rotavírus, este gene pode ser usado para a detecção de vários sorotipos, sendo que o iniciador gênico normalmente utilizado flanqueia uma região de 379 pb (pares de base) neste gene. Posteriormente, o sorotipo específico pode ser determinado com iniciadores para os genes codificadores de VP7 e VP4 (ITURRIZA-GÓMARA et al., 2002).

A principal forma de transmissão do rotavírus é pela via feco-oral, replicando-se primeiramente no trato gastrointestinal. A probabilidade de infecção pela exposição a uma partícula viral de rotavírus é de 31% e não mais do que 1 PFU (unidade formadora de foco) é requerida para causar infecção em 1% de adultos que não possuem anticorpos para este vírus (FONG; LIPP, 2005). A veiculação através da água tem sido evidenciada durante todas as estações, porém com maior frequência durante o inverno (BORCHARDT et al. 2003; KAPIKIAN; CHANOCK, 1996; MEHNERT et al., 1997).

Números limitados de casos de rotaviroses relacionados com o consumo de águas contaminadas têm sido publicados, sendo que a maioria foi resultante da ingestão de águas de beber contaminadas por descargas de esgoto (FROST et al., 2002; GOFTI-LAROCHE et al., 2001; LOPMAN et al., 2003).

A diversidade genética dos RV parece ser mais frequente nos países em desenvolvimento, provavelmente devido aos baixos níveis de higiene, defesa imunológica limitada, infecções parasitárias concomitantes, desnutrição, além do estreito relacionamento entre o homem, animais domésticos e outros animais, proporcionando, assim, infecções mistas e, consequentemente, maior possibilidade de rearranjos genômicos (COOK et al., 2004).

Os RV constituem um dos principais agentes etiológicos de diarréia no mundo. Anualmente, somente os RV do sorogrupo A são responsáveis por 111 milhões de episódios de gastrenterite 25 milhões de visitas ambulatoriais, 2 milhões de hospitalizações e 352 a 592.000 mortes de crianças de zero a cinco anos, principalmente, na África, América Latina e Ásia (PARASHAR et al., 2003).

Os RV foram detectados pela primeira vez, no Brasil em 1976, a partir da microscopia eletrônica de fezes diarréicas de crianças em Belém (PA). Um estudo conduzido em hospitais brasileiros registrou a prevalência de diarréia por rotavírus de 12 a 42%. Os sorotipos G1, G2, G3 e G4 representaram cerca de 2/3 das amostras circulantes no país; sabe-se que o sorotipo G5, genótipo P[8], está associado a, no mínimo, 10% das diarréias, e que 70% das crianças adquirem anticorpos já aos 4-5 anos de idade (LINHARES et al., 2001).

1.4. Norovírus

A família Caliciviridae é formada por quatro grupos de vírus genética e antigenicamente distintos (*Lagovirus*, *Vesivirus*, *Norovirus* e *Saporovirus)*. Os calicivírus humanos estão agrupados nos gêneros *Norovirus e Sapovirus.*

Os vírus pertencentes a esta família apresentam simetria icosaédrica, RNA fita simples, de polaridade positiva codificando 3 ORFs. Medem cerca de 30 a 38 nm de diâmetro com 32 depressões na superfície, em forma de “cálice”, arranjadas em simetria icosaédrica, sendo não envelopados (JIANG et al., 1993).

A diversidade genética e antigênica dos norovírus (NoV) inclui dois genogrupos (I e II) subdivididos em genótipos e, embora não exista consenso para esta subclassificação existem sete tipos genéticos descritos do genogrupo I (GGI) e oito do genogrupo II (GGII) (BON et al., 2004).

O diagnóstico de infecções por calicivírus humanos é dificultado por não existir uma forma eficiente de isolamento desses vírus em cultivo celular. Desta forma, inviabiliza-se o isolamento e amplificação das partículas virais *in vitro* e/ou *in vivo*. A literatura científica tem relatado a possibilidade de se utilizar os norovírus murinos (MNV) como substitutos dos humanos (não adaptados ao cultivo *in vitro*) para estudos de estabilidade e quantificação *in vitro* destes vírus causadores de diarréias severas em adultos e crianças (BAERT et al., 2008). O desenvolvimento de um ensaio imunoenzimático comercial para detecção de norovírus em fezes poderia contribuir para a vigilância epidemiológica destes vírus, embora resultados negativos durante um surto devam ser processados pela reação de polimerase em cadeia precedida de transcrição reversa, que ainda é o método mais sensível de detecção (BON et al., 2004).

A gastroenterite viral aguda causada pelos NoV é branda e autolimitada, seus sintomas são basicamente vômitos e diarréia e aparecem de 20 a 50 horas após o consumo de água e/ou alimentos contaminados e duram de 1 a 8 dias (JOHANSSON et al., 2002). Dados epidemiológicos demonstraram uma predominância desta doença durante o inverno (APPLETON, 2000; BORCHARDT et al., 2003; KAPIKIAN et al., 1996; PRINGLE, 1999).

Estudos de prevalência dos NoV têm sido conduzidos em diferentes continentes; entretanto, os dados disponíveis são principalmente de países desenvolvidos, que indicam que estes vírus são responsáveis no mundo por 80-90% dos surtos de gastrenterites não bacterianas (KIRKWOOD; BISHOP, 2001). Em países em desenvolvimento, a prevalência de NoV é subestimada ou desconhecida, principalmente em comunidades de baixa renda (XAVIER et al., 2009). No Brasil, os poucos estudos apontam uma prevalência de 8,6 a 33,3% entre crianças hospitalizadas (SOARES et al., 2007).

Estudos têm demonstrado que os calicivirus, especialmente os Norovirus, tem se tornado um crescente problema de saúde pública em todo o mundo, sendo comumente responsáveis por surtos associados a comunidades - hospitais, creches, escolas, asilos e cruzeiros (CARRIQUE-MAS et al., 2003; GALLIMORE et al., 2004a, b ; MONROE et al., 2000). Existem também evidências de surtos de veiculação hídrica, através da exposição a

águas de fins recreacionais, de tanques, de sistema de água industrial, água de balneários e até mesmo de águas potáveis (FROST et al., 2002; LEE et al., 2002; NYGARD et al 2003).

Estes vírus têm a habilidade de sobreviver a altos níveis de cloração da água e variações de temperaturas, facilitando sua transmissão através de águas para consumo e de recreação, gelo comercial e alguns alimentos como ostras cozidas e cruas (CANNON et al., 1991; KIRKLAND et al., 1996).

1.5. Vírus da Hepatite A

O vírus da hepatite A (HAV), pertencente ao gênero *Hepatovirus* e à família Picornaviridae, é formado por um nucleocapsídeo icosaédrico não envelopado (Figura 4), medindo de 27 a 32 nm de diâmetro. O genoma é uma molécula de RNA linear com 7,5 Kb, de fita simples e polaridade positiva. Esse genoma é dividido em três regiões: 5' não codificadora; longa ORF codificadora de 11 proteínas, algumas estruturais (VP1, VP2, VP3, VP4, 2A, 3B) e outras não estruturais (2B, 2C, 3A, 3C, 3D) e 3' não codificadora.

É um vírus com genoma bastante conservado, sendo que as diferentes cepas do HAV do mundo inteiro correspondem a um único sorotipo, que são classificadas em seis genótipos, com base em análises filogenéticas das sequências dos nucleotídeos da região VP1/2A (LU et al., 2004; ROBERTSON et al., 1992). A maioria das cepas humanas estudadas pertence aos genótipos I ou III (ROBERTSON et al., 1992).

O isolamento primário destes vírus em culturas celulares é difícil, lento e fastidioso; o vírus selvagem não apresenta efeito e tende a estabelecer uma infecção persistente. O HAV é extremamente resistente, pode sobreviver por aproximadamente um mês à temperatura ambiente, e somente é parcialmente degradado após 12 horas a 60ºC (CROMEANS; SOBSEY; FIELDS, 1987).

O HAV é adquirido primariamente pela rota feco-oral (HOLLINGER; TICEHURST, 1996), pelo contato pessoa-a-pessoa ou pela ingestão de água e alimentos contaminados. Via trato-alimentar, chega à corrente sanguínea e espalha-se para infectar as células parenquimais do fígado. O vírus replica-se nos hepatócitos e células de Kupfer, é excretado na bile e eliminado nas fezes, podendo manifestar-se como hepatite ictérica ou anictérica, ou desenvolver-se de forma inaparente ou subclínica, o que acontece em 80% dos casos (TAVARES et al., 2005).

A distribuição mundial da hepatite A apresenta diferentes níveis de endemicidade e está diretamente relacionada com as condições sanitárias e socioeconômicas das populações.

Em torno de 20 a 25% dos casos de hepatite clinicamente sintomáticas no mundo inteiro e aproximadamente 1,4 milhões de casos de hepatites são relacionados com o HAV (STRADER et al., 1996). A doença é hiperendêmica em países em desenvolvimento da Ásia, África e América do Sul e Central, onde há superpopulação, inadequado saneamento básico e baixas condições de higiene. Onde pobreza e carência são extremas, a infecção, geralmente subclínica é adquirida na infância e assim os adultos possuem anticorpos protetores. A maioria dos casos clínicos é observado em crianças e adultos jovens e em visitantes de países mais desenvolvidos. A maioria dos surtos ocorre principalmente quando poços e águas de consumo são contaminadas com esgotos. Estudos já demonstraram a presença destes vírus em água de esgoto (MORACE et al., 2002; VAIDYA et al., 2002), de rios (TAYLOR et al., 2001) e do mar (BROOKS; GERSBERG; DHAR, 2005). A presença de HAV nestas águas é uma preocupação de saúde pública, uma vez que mesmo em baixa concentração, HAV pode causar doença e pode permanecer de 12 a 10 meses na água (CUTHBERT, 2001).

O Brasil, assim como outros países em desenvolvimento, sempre foi considerado um país de alta endemicidade para o HAV, pois dados epidemiológicos demonstram que aproximadamente 90% da população apresentam anticorpos anti-HAV e que a maioria das crianças já era imune aos 10 anos de idade. A grande disparidade socioeconômica entre a população reflete diretamente numa grande diversidade na prevalência de infecção pelo HAV, variando entre 56% nas regiões Sul e Sudeste e até 90% na região Norte (CARRILHO et al., 2005).

Na região Sul, num estudo realizado por Ferreira et al. (2002) a prevalência de anticorpos anti-HAV encontrado foi entre 4,7% e 9,5%. As variações encontradas no Brasil mostram que, ao mesmo tempo em que a Hepatite A é endêmica no nosso meio, há um número crescente de pessoas suscetíveis para infecção pelo HAV o que pode levar à ocorrência de surtos ou casos esporádicos. Sendo assim a infecção pelo HAV continua sendo a forma mais comum dentre as hepatites virais agudas (VILLAR, 2006).

1.6. Concentração, detecção, quantificação e determinação da viabilidade de vírus entéricos em amostras ambientais.

O controle da contaminação de vírus no ambiente é um processo complexo, pois se encontram dispersos em grandes volumes de água, adsorvidos a biosólidos ou em outros tipos de amostras ambientais. Além da baixa concentração, a qualidade da amostra ambiental a ser

testada também é um fator negativo devido à frequente presença de inibidores de reações enzimáticas, que sao utilizadas para a detecção dos genomas virais.

Atualmente, não existem métodos padronizados de detecção de vírus em águas ou em alimentos, em virtude de não ser uma prática rotineira nos laboratórios de microbiologia. Em diferentes investigações realizadas, os procedimentos de concentração de vírus a partir de quantidades volumosas de amostras de água geralmente incluem a etapa de adsorção-eluição, utilizando filtros ou membranas carregadas positiva ou negativamente, fibras, pó ou lã de vidro, hidróxido de alumínio e ultrafiltração por fluidez tangencial. Estas etapas são seguidas por precipitação através de floculação orgânica ou inorgânica, reação com sulfato de amônio, hidroextração com polietilenoglicol e/ou ultracentrifugação (BEURET, 2003; BORCHARD et al., 2003; BOSCH, 1998; CALGUA et al., 2008; FORMIGA-CRUZ et al., 2002; KATAYAMA et al., 2002; KELLER; GONÇALVES, 2001; KITTIGUL et al., 2001; PINA et al., 1998; PUIG et al., 1994; QUEIROZ et al., 2001; SOBSEY; JONES, 1979).

A carga elétrica dos vírus se altera em função do pH, composição e força iônica da solução a ser usada na eluição. Os métodos de adsorção-eluição mais comumente utilizados são os que interagem com as cargas dos vírus presentes na água, através de mudanças de pH (FONG; LIPP, 2005). Geralmente, a água a ser processada é previamente clarificada para evitar que os materiais em suspensão impeçam a concentração viral. No caso do método que utiliza membrana negativa descrito por Katayama et al. (2002), após a clarificação da água ela é acidificada e adicionada de uma solução doadora de cátions, como por exemplo, cloreto de magnésio ou cloreto de alumínio, favorecendo a adsorção dos vírus. Após a adsorção, os vírus são eluídos da membrana com uso de soluções alcalinas, tal como a de o hidróxido de sódio. Entretanto a recuperação viral a partir de diferentes tipos de água nem sempre é a mesma devido ao fato da adsorção dos vírus às membranas negativas ser influenciada por sais, cátions multivalentes ou condições ácidas (HARAMOTO et al., 2005, 2006, 2007; RIGOTTO et al., 2009; VICTORIA et al., 2009).

No Brasil, desde 1988, trabalhos realizados têm utilizado um método de concentração em duas etapas: adsorção-eluição por filtração através de membrana de filtro eletropositiva Zeta Plus 60 S (ZP60S), seguida de ultracentrifugação, para a pesquisa de rotavírus, adenovírus e vírus da hepatite A em amostras de água (MEHNERT et al., 1997; QUEIROZ et al., 2001). Esse método é simples, rápido e de baixo custo, (quando a ultracentrífuga estiver disponível), entretanto, apresenta a desvantagem da utilização de extrato de carne como eluente viral, que por ser rico em substâncias orgânicas, pode inibir a reação de amplificação gênica interferindo com a detecção viral, após sua concentração.

Outros métodos de concentração também têm sido utilizados no Brasil, como a filtração em membrana estéril e precipitação com polietilenoglicol 6000 para pesquisa de rotavírus em águas de esgoto (KELLER; GONÇALVES, 2001). Após o desenvolvimento do método de concentração em membrana negativa pelo grupo de Katayama e colaboradores em 2002, esse método tem sido aplicado no Brasil na detecção do vírus da hepatite A, astrovírus e norovírus em diversas amostras ambientais (DE PAULA et al., 2007; MIAGOSTOVICH et al., 2008; VICTORIA et al., 2009; VILLAR et al., 2007).

Dentre os métodos de detecção de vírus entéricos em amostras ambientais, os métodos imunológicos, tais como ELISA, imunofluorescência, imunoperoxidase, quimioluminescência e citometria de fluxo destacam-se como técnicas altamente específicas usadas no acompanhamento de processos de recuperação e detecção de vírus isolados em culturas de células a partir de amostras de água (WYN-JONES; SELLWOOD, 2001).

As culturas de células são métodos tradicionais utilizados na detecção de alguns vírus, como os enterovírus, em amostras concentradas de água. Apesar de serem muito sensíveis e detectarem partículas virais infecciosas, requerem de três dias a mais de seis semanas para que os efeitos citopáticos virais sejam evidenciados, além de serem uma técnica onerosas e com baixa especificidade (FONG; LIPP, 2005; TAVARES, 2005).

O uso de ensaios moleculares, particularmente o da reação em cadeia da polimerase (PCR), tem permitido novos avanços na detecção e controle de vírus entéricos em águas. No entanto, uma desvantagem é a suscetibilidade do ensaio aos inibidores (cátions divalentes, ácidos húmicos, metais, etc.) comumente encontrados em ambientes aquáticos (FONG; LIPP, 2005).

As técnicas tradicionais de separação de inibidores dos ácidos nucléicos utilizam extração orgânica com fenol e clorofórmio. A desvantagem desta técnica é a perda de consideráveis quantidades do genoma viral durante as etapas de repetitivas lavagens. Shieh e colaboradores (1995) utilizaram a técnica que emprega o isotiocianato de guanidina, desenvolvida por Boom et al. (1990), para a extração do genoma de enterovírus em amostras de esgoto e eliminaram substâncias inibidoras aumentando a proporção de amostras positivas de 3 para 7 dentro das 11 amostras analisadas. *Kits* comerciais que visam à extração de ácidos nucléicos de amostras ambientais também têm sido desenvolvidos por várias empresas, tais como os da Qiagen.

Variações na técnica de PCR qualitativo têm sido empregadas para detectar vírus em estudos ambientais e clínicos. A *nested* PCR é uma das mais empregadas para detecção de vírus entéricos em amostras de água, aumentando a sensibilidade da reação, por introduzir

uma etapa extra de amplificação gênica. Nesta reação ocorre uma amplificação do produto gerado na primeira reação, através da utilização de iniciadores, que flanqueiam uma sequência interna deste amplicon. Isto também auxilia na remoção de inibidores presentes na amostra. Esta técnica já foi utilizada por diversos autores, alcançando limites de detecção de até uma partícula viral por amostra testada (KITTIGUL et al., 2005; PUIG et al., 1994; RIGOTTO et al., 2005).

Uma das limitações das técnicas moleculares é a incapacidade de determinar a viabilidade dos vírus. A integração da cultura celular com a PCR (ICC-PCR) é uma forma alternativa e eficiente, rápida e específica, capaz de produzir informações sobre a infectividade viral (KO et al., 2005; REYNOLDS et al., 1996, 2004; RIGOTTO et al. 2005; RUTJES et al., 2009), sendo que seus inconvenientes são a dificuldade de adaptá-la a estudos em larga escala e à automoção. Entretanto, esta técnica pode ser mais rápida e mais sensível que a cultura celular tradicional, uma vez que está acoplada a PCR aumentando desta forma a remoção dos inibidores e a sensibilidade da técnica.

Os métodos de quantificação de viral, tal como PCR quantitativo ou em tempo real, têm sido cada vez mais utilizados mundialmente. Este em particular, pode ser considerado de alta sensibilidade para a detecção rápida e quantificação precisa de organimos patogênicos em amostras clínicas e ambientais (ALBINANA-GIMENEZ et al., 2009; BROOKS; GERSBERG; DHAR, 2005; HARAMOTO et al., 2007; KO et al., 2005).

Os métodos de concentração e detecção de vírus em amostras ambientais ainda apresentam muitas divergências, portanto é importante que eles continuem sendo desenvolvidos e testados de modo que permitam a detecção adequada de patógenos virais veiculados pela água. Cada vez mais almeja-se de um protocolo padrão que seja simples, eficiente e acessível para ser utilizado na rotina da avaliação virológica de águas, como também para o estabelecimento de indicadores para o monitoramento da presença de vírus patogênicos no ambiente. Isso iria contribuir significativamente para a qualidade sanitária do ambiente aquático, em termos de segurança virológica e traria consequências positivas para a saúde dos seres vivos, já que a água é a principal fonte de sobrevivência de todas as espécies da terra.

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3. OBJETIVOS

Objetivo Geral: Padronizar metodologias moleculares e de cultura celular para implementar o monitoramento de águas ambientais e amostras de ostras, no que se refere aos métodos de concentração, detecção, quantificação e determinação da viabilidade de vírus entéricos.

Objetivos específicos

- Padronizar metodologias de concentração, detecção, quantificação e determinação da viabilidade de vírus entéricos (adenovírus, rotavírus, norovirus, e vírus da hepatite A) no ambiente aquático (especificamente em águas ambientais e ostras de cultivo).
- Realizar a pesquisa de adenovírus humanos, norovírus dos genogrupos GI e GII, rotavírus humanos do grupo A e vírus da hepatite A em águas ambientais e ostras de cultivo, durante o período de Junho de 2007 a Maio de 2008 através das metodologias padronizadas.
- Realizar o estudo de decaimento do infectividade de adenovírus humanos em amostras de águas mantidas em diferentes temperaturas, por períodos de tempo variáveis.

4. MATERIAIS E MÉTODOS & RESULTADOS

As metodologias aplicadas para o alcance dos objetivos propostos, bem como os resultados obtidos durante este doutoramento, serão apresentados, na forma de apêndices, como capítulo de livro publicado, e artigos aceitos ou segundo as regras submetidos à publicação em revistas científicas indexadas e serão listados a seguir na ordem que as publicações foram submetidas. Cada uma das publicações constituirá um capítulo da presente tese.

1. **RIGOTTO, C.;** CORREA, A.A.; KOLESNIKOVAS, C.K.M.; ALBARNAZ, J.D.; SIMÕES, C.M.O.; BARARDI, C.R.M. Detection of Adenoviruses and Hepatitis A virus in water samples and oysters: use of three different nucleic acid extraction methods. In: **Trends in Water Resources Research**. Nova Publishers, New York, 2008. p. 121-137.

2. **RIGOTTO, C.**; KOLESNIKOVAS, C.K.M.; MORESCO, V.; SIMÕES, C.M.O.; BARARDI, C.R.M. Evaluation of HA negatively charged membranes in the recovery of human adenoviruses and hepatitis A virus in different water matrices. **Memórias do Instituto Oswaldo Cruz**. Rio de Janeiro, Vol. 104(7): 970-974, November 2009. Fator de Impacto: 1,225.

3. SCHLINDWEIN, A.D.; **RIGOTTO, C.**; SIMÕES, C.M.O.; BARARDI, C.R.M. Detection of enteric viruses in sewage sludge and treated wastewater effluent. **Water Science and Technology**. *Aceito para publicação em Outubro de 2009*. Fator de Impacto: 1,005.

4. VICTORIA, M.; **RIGOTTO, C.**; MORESCO, V.; CORRÊA, A.A.; KOLESNIKOVAS, C.K.M.; LEITE, J.P.; MIAGOSTOVICH, M.P.; BARARDI, C.R.M. Assessment of norovirus contamination in environmental samples from Florianópolis City, Southern Brazil. **Journal of Applied Microbiology**. Published Online: Jan 4 2010 3:22PM DOI:10.1111/j.1365-2672.2009.04646.x. Fator de Impacto: 2,028.

5. **RIGOTTO, C.**; VICTORIA, M.; MORESCO, V.; KOLESNIKOVAS, C.K.M.; CORREA, A.A.; SOUZA, D.S.M.; MIAGOSTOVICH, M.P.; SIMÕES, C.M.O.; BARARDI, C.R.M. Assessment of adenovirus, hepatite A virus and rotavirus contamination in environmental samples in Florianópolis, South Brazil. **Water Research**. *Submetido em Setembro de 2009*. Fator de Impacto: 4,274.

6. **RIGOTTO, C.**; HANLEY, K.; ROCHELLE, P.A.; DE LEON, R.; BARARDI, C.R.M.; YATES, M.V. Survival of adenoviruses serotypes 41 and 2 in surfaces and ground waters measured by a plaque assay. *Artigo em fase de finalização.*

Capítulo 1

Capítulo de livro: RIGOTTO, C.; CORREA, A.A.; KOLESNIKOVAS, C.K.M.; ALBARNAZ, J.D.; SIMÕES, C.M.O.; BARARDI, C.R.M. Detection of Adenoviruses and Hepatitis A virus in water samples and oysters: use of three different nucleic acid extraction methods. In: **Trends in Water Resources Research**. Nova Publishers, New York, 2008. p. 121-137.

Capítulo 2

RIGOTTO, C.; KOLESNIKOVAS, C.K.M.; MORESCO, V.; SIMÕES, C.M.O.; BARARDI, C.R.M. Evaluation of HA negatively charged membranes in the recovery of human adenoviruses and hepatitis A virus in different water matrices. **Memórias do Instituto Oswaldo Cruz**. Rio de Janeiro, Vol. 104(7): 970-974, November 2009. Fator de Impacto: 1,225

Capítulo 3

SCHLINDWEIN, A.D.; **RIGOTTO, C.**; SIMÕES, C.M.O.; BARARDI, C.R.M. Detection of enteric viruses in sewage sludge and treated wastewater effluent. **Water Science and Technology**. *Aceito para publicação em Outubro de 2009.* Fator de Impacto: 1,005.

Mensagem encaminhada: Mensagem Encaminhada: WST-WSTWS-EM09567 - please submit revised manuscript
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Para: cbarardi@ccb.ufsc.br
Water Science and Technology
Article title: DETECTION OF ENTERIC VIRUSES IN SEWAGE SLUDGE AND TREATED WASTEWATER EFFLUENT
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Dear Dr. Barardi,

Following the peer review of your initial submission, I am pleased to inform you that your article

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DETECTION OF ENTERIC VIRUSES IN SEWAGE SLUDGE AND TREATED WASTEWATER EFFLUENT

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ABSTRACT

Sewage sludge and treated wastewater when contaminated with enteric virus and discharged into the environment, could pose a human health risk. The aim of study was to verify the presence and viability of enteric viruses in sewage sludge and treated wastewater at a local sewage plant in Florianopolis city, Brazil. Sewage sludge was concentrated by organic flocculation and polyethylene glycol precipitation and wastewater by electronegative membrane filtration and ultrafiltration by Centriprep Concentrator. Adenovirus (AdV), hepatitis A virus (HAV), and Rotavirus (RV) were searched during twelve months in all samples and Poliovirus (PV) was also included in sewage sludge. AdV was the most prevalent in both kind of samples, followed by RV, PV (in sludge) and HAV. Viral viability by cell culture (ICC-PCR) was: AdV: 100%, HAV: 16.7%, PV: 91.7%, RV: 25% in sludge and AdV: 66.6%, HAV: 66.6% and RV: 0% in wastewater. IFA for AdV in sludge ranged from 70 to 300 FFU/ml. QPCR for AdV ranged from $4.6x10^4$ to $1.2x10^6$ and from 50 to $1.3x10^4$ gc/ml in sludge and wastewater, respectively. HAV quantification in sludge ranged from $3.1x10^2$ to $5.4x10^2$. In conclusion, it was possible to correlate presence and viability of enteric viruses in the environmental samples analyzed.

Key words: sewage sludge, treated wastewater, enteric viruses, molecular techniques, cell culture.

INTRODUCTION

Sludges derived from wastewater consist of organic matter that can be used for agricultural purposes as fertilizers. However, they may contain numerous pathogenic microorganisms, mostly of fecal origin. The presence of bacteria, viruses and parasites in sewage sludge implies a potential health threat. The agricultural use, by spreading on the land, is the principal vehicle by which sludge is disposed of and can only be of lasting value if the sludge has undergone treatment by biological, chemical, thermal, or other suitable processes to diminish its capacity for fermentation and eliminate any health risk related to such use (Monpoeho et al. 2004).

It has been previously reported several waterborne outbreaks associated with water even when it has met all bacteriological standards. Routine tests for bacterial pathogen contamination are proved to be inconsistent indicators of viral contamination (Chapron et al. 2000).

PCR detection can be sensitive and specific. The efficiency of viral amplification from environmental samples by PCR is influenced by the ability to recover the virus from the environmental matrix and the purity of the recovered nucleic acid (Fong and Lipp, 2005).

Integrated cell culture-PCR (ICC-PCR) overcomes the individual disadvantages of cell culture and PCR. The use of cell culture helps to dilute out PCR inhibitors and provides an *in vitro* amplification system which increases the number of viruses differentiating between infectious and non-infectious virus (Reynolds, 2004).

Immunofluorescence staining is also a very useful test for the detection of viral replication in tissue culture and considered more reliable than the plaque assay. Using an immunofluorescence assay, virus infection can be detected even when the CPE is not present (Birch et al. 1983).

In the present study, an association of molecular methods, integrated cell culture-molecular methods and immunofluorescence was applied to detect human enteric viruses in sewage sludge and treated wastewater effluent from the sewage plant in Florianópolis, SC, Brazil, which uses activated sludge as a treatment process.

METHODS

Virus and cell cultures

HAV-cytophatic strain HAF 203, Human AdV 2 (AdV2) (genogroup C, serotype 2) and Human AdV 5 (AdV5) (genogroup C, serotype 5) were respectively propagated in a continuous line of fetal FRHK-4 cells (rhesus kidney-derived cells), A549 (carcinomic human alveolar basal epithelial cells) and Hep-2 cells (human larynx carcinoma).

Poliovirus type 2 (PV2) and Simian RV SA11 (group A, serotype G3) were respectively propagated in VERO cells (an established line of African green monkey kidney fibroblasts) and MA104 cells (a continuous line of fetal rhesus kidney cells).

Cells were cultured in Eagle's minimal medium (MEM-Sigma), supplemented with 5% (v/v) fetal bovine serum (FBS) (Gibco-BRL), streptomycin (100µg/ml), penicillin G (100U/ml), and amphotericin (0.025µg/ml) (Gibco-BRL). The Hep-2 cells had been kept in the same conditions cited above, however supplemented with 1% (v/v) of FBS.

For determination of virus titers, indirect immunofluorescense assay (IFA) was used for AdV2, RV and HAV viruses, as previously described with modifications (Barardi et al. 1999) and plaque assay was the method of choice for AdV5 and PV2 titer determination (Burlenson et al. 1992). The titer of the AdV2 was 6.4 x 10^9 focus forming units (FFU)/ml, AdV5 was 7.0 x 10^6 plaque forming units (PFU)/ml, HAV was 4.5 x 10^5 FFU/ml, PV2 was 4.7 x 10^5 PFU/ml and RV was 6.0 x 10^6 FFU/ml.

Activated sludge and treated wastewater samples

A total of 24 samples - 12 activated sludge sample and 12 treated wastewater effluent - were collected monthly over a period between June 2007 to May 2008 from the wastewater treatment plant. All samples (2 l of wastewater and 5.0 ml of sludge) were kept at 4°C in sterile containers and processed within 24 h of collection.

Virus concentration method for activated sludge

The technique for virus elution from sludge samples was described by the Environmental Protection Agency (EPA 1992) and was previously modified by our group (Schlindwein et al. 2009). For virus concentration PEG 6000 precipitation was employed as

described by Lewis and Metcalf (1988). The supernatant was discarded and the resulting pellet was suspended in 5.0 ml of 0.1M phosphate buffer (pH 7.2).

Decontamination step using chloroform was performed as described by Mignotte et al. (1999).

Nucleic acid extraction for activated sludge samples

For DNA/RNA extraction, 500 µl samples of processed sewage sludge were used as previously described by Schlindwein et al. (2009). DNA was extracted from all AdV5 seeded samples using the phenol:chloroform:isoamyl alcohol method, according to Sambrook and Russell (2001). The nucleic acids were suspended in 50 µl of sterile MilliQ water.

RNA was purified from the HAV, RV and PV2 seeded samples with Trizol® LS (Invitrogen Life Technologies) according to the manufacturer's instructions. The nucleic acids were suspended in 50 µl of sterile diethylpyrocarbonate (DEPC)-treated water. Purified nucleic acids were stored at -80°C for the RT-PCR assays.

Virus concentration method for treated wastewater samples

Concentration of viruses in wastewater was performed by adsorption in an electronegative membrane and subsequent elution as described by Katayama et al. (2002). Four hundred microliters of the water concentrate (2 ml) was further used for nucleic acid extraction.

Nucleic acid extraction for wastewater samples

Viral nucleic acids were extracted by the procedure described by Boom et al. (1990). This procedure uses guanidinium thiocyanate and adsorption of the nucleic acids to silica particles.

PCR and nested PCR for AdV detection

AdV DNA was detected in samples by using the oligonucleotide primers pair hexAA 1885 /hexAA 1913 and nexAA 1893/nexAA 1905 described by Allard et al. (1992). The expected size of the PCR product was 300 bp and 142 bp for nested PCR.

Reverse transcription (RT-PCR) or nested RT-PCR to detect HAV, PV and RV

Conventional RT-PCR was performed using random and specific primers for reverse transcription and genome amplification. Random hexamers primers were purchased from Promega (Brazil) and were used for cDNA synthesis. Briefly, a 5.0 µl aliquot of RNA was heated at 99°C for 5 min, followed by quick chilling on ice for 2 min. The denatured RNA was added to a mixture containing random primer, 50mM Tris-HCl, pH 8.4, 75mM KCl, 0.5mM of each dATP, dCTP, dTTP and dGTP, 20U of RNAse inhibitor and 100U of M-MLV reverse transcriptase (all reagents were purchased from Promega), in a 25 µL volume. Reverse transcription of viral genomic RNA was carried out at 37°C for 60 min.

HAV RNA was detected in sewage sludge and wastewater samples by nested RT-PCR, using the oligonucleotide primers pair F6 (+) and F7 (-), which amplifies a 392 bp fragment, suitable to amplify all HAV genotypes (‘universal primers’). Internal primers were F8 (+) and F9 (-), which amplifies a 247 bp fragment (De Paula et al. 2004).

PV2 RNA was detected in sewage sludge samples by RT-PCR, using the oligonucleotide primers pair Polio-R and Polio-L, which amplifies a 394 bp fragment from the 5’ non-coding region (Atmar et al. 1993).

RV RNA was detected in sewage sludge and wastewater samples by RT-PCR, using the oligonucleotide primers pair VP6-F (+) and VP6-R (-), which amplifies a 379 bp fragment of the VP6 gene (Iturriza-Gómara et al. 2002).

Quality Control

To avoid false positives resulting from carryover contamination of amplified virus particles or viral nucleic acid, separate areas and equipment were used for each stage of the process. Negative controls (non spiked autoclaved water) and positive controls (virus suspensions) were included with each set of test samples and taken through nucleic acid extraction and enzymatic amplification assays. Additional blank controls (containing the same reaction mixture except for the nucleic acid template) were incorporated with all PCR assays.

Cytotoxicity assays

FRHK-4, A549, VERO and MA104 cell lines were assayed for sewage sludge and FRHK-4, A549 and MA104 for wastewater. Cytotoxic assays were performed according to

described by Rigotto et al. (2005) using 2-fold serial dilution (1:2 to 1:32) in serum-free MEM of unseeded autoclaved sewage sludge and treated wastewater.

Plates were incubated and observed for cytotoxic effects after 48h for MA104 and 72h for A549 and Vero cells; and up to a week for FRhK-4 cells. Each observation was compared with negative controls containing only normal cell monolayer and medium. After period of incubation, the cellular integrity was confirmed by removing the medium by suction and staining with 250 µl of naphthalene black (Sigma). The cells were analyzed to establish the first non-cytotoxic dilution of sewage sludge and treated wastewater to be used at ICC-PCR and IFA assays.

Integrated cell culture PCR (ICC-PCR)

For the ICC-PCR assay, FRHK-4, A549, VERO and MA104 cells monolayers were prepared in 24-well microplates. The sewage sludge samples, treated wastewater and the positive controls (viral suspensions) were diluted in maintenance medium. Inocula were then allowed to adsorb to previously PBS washed cell-layer during 90 min at 37°C. Non-adsorbed viruses were removed from cell monolayers by three times washing with PBS. After incubation as described above, plates were submitted to three cycles of freeze/thaw for cell lysis and virus releasing. The supernatant was recovered and 0.5 ml was used for viral genome isolation and PCR detection.

IFA assay for AdV detection in sewage sludge

The IFA assay was performed to confirm the ICC-PCR results for AdV5according to Barardi et al. (1999) with minor modifications. This virus was selected due to its high stability in several environments and resistance to some disinfection treatments.

The same procedure was carried through with the viral fluid of AdV2 which was diluted serially from 10^{-1} to 10^{-7} in autoclaved sewage sludge samples and 100 µl of each dilution was inoculated in the wells in duplicate, in order to observe if the sewage sludge sample was capable to inhibit the replication viral response of the AdV in A549 cells.

Quantification of AdV DNA in sewage sludge and treated wastewater by Taqman PCR

Samples containing viable AdV particles as showed by ICC-PCR assays, were quantified by real time PCR. Amplification was performed in a 25 µl reaction mixture with

the PCR Master Mix (Applied Biosystems). The reaction consisted of 10 µl of a DNA sample or 10 µl of a quantified AdV41 cloned in pBR322 (kindly donated by R. Girones, Barcelona University). TaqMan master mix, the corresponding primers and TaqMan probe at their corresponding concentrations. AdV genomes were quantified with 0.9 µM the primers AdF and AdR and 0.225 µM of the AdP1 probe described by Hernroth et al. (2002). All samples were run in duplicate which four replies and positive and negative controls were included. The amount of DNA was defined as the average of the data obtained.

Quantification of HAV RNA in sewage sludge and treated wastewater by Taqman RT-PCR

Samples containing viable HAV particles as showed by ICC-PCR assays, were quantified by real time RT-PCR. Amplification was performed in a 25 µl reaction mixture with the kit Quantitec® Probe RT-PCR (Qiagen®). The reaction contained 10 µl of a RNA sample or 10 µl of a quantified pGEM-T easy vector with HAV insert (kindly donated by R. Girones, Barcelona University), TaqMan master mix, and the corresponding primers and TaqMan probe at their corresponding concentrations. HAV genomes were quantified with 0.25 µM the primers HAVF and HAVR and 0.15 µM of the HAVP probe described by Jothikumar et al. (2005). The amount of RNA was defined as the average of the data obtained.

RESULTS AND DISCUSSION

PCR analysis

Traditional PCR was applied for nucleic acids extracted from sewage and wastewater samples in order to detect the presence or absence of the enteric viruses genomes on these samples. All sewage samples (100%) and 75% of treated wastewater samples tested positive for the presence of AdV genome when nested PCR was applied. For HAV genome presence using nested RT-PCR, 3 of 12 samples (25%) were positive both in sludge and wastewater samples. The RT-PCR analysis performed with RV-specific primers identified 4 of 12 sludge samples (33%) and 5 of 12 wastewater samples (41.6%) positive for RV RNA sequences. For PV in sludge samples, 8 of 12 (66.7%) were positive (Table 1).

Table 1 Detection of enteric viruses in sewage sludge and treated wastewater samples by PCR.

Sampling (month/year)	AdV		HAV		PV	RV	
	Sewage Sludge	Treated Wastewater	Sewage Sludge	Treated Wastewater	Sewage Sludge	Sewage Sludge	Treated Wastewater
06/2007	+	-	+	-	+	-	-
07/2007	+	-	-	-	+	+	+
08/2007	+	+	+	-	-	+	-
09/2007	+	-	-	-	+	+	-
10/2007	+	+	-	+	+	-	+
11/2007	+	+	-	+	+	-	+
12/2007	+	+	-	-	-	-	+
01/2008	+	+	-	+	-	-	+
02/2008	+	+	+	-	+	+	-
03/2008	+	+	-	-	-	-	-
04/2008	+	+	-	-	+	-	-
05/2008	+	+	-	-	+	-	-

+: positive sample. -: negative sample.

Environmental samples, especially urban sludge, contain many organic and inorganic compounds (humic acids, polyphenols and heavy metals) that are toxic and might cause lysis in cell culture. These compounds are also responsible for forming complexes with nucleic acids and inhibit the enzyme amplification.

In order to ensure specificity of detection, eliminate any false-positive results, and increase the amplification signal, nested PCR amplification was applied for the detection of AdV and HAV genomes in all samples. This strategy allowed us to detect a small number of viral contaminants on sludge and wastewater samples. The high percentage of samples positive for AdV distributed over the period of study (June 2007 to May 2008) indicates that there is no occurrence of seasonality. These results are consistent with reports from Formiga-Cruz et al. (2005) which found that AdV are excreted throughout the year in higher numbers than enteroviruses and HAV. The results for AdV reported support the hypothesis that AdV is a suitable index for human viral environment contamination. AdV is more stable in various environments when compared with other enteric viruses, and it is more resistant to some disinfection treatments (UV irradiation and chlorine). These results corroborate with those described by Pina et al. (1998) and Formiga-Cruz et al. (2005) which showed that the AdV are the most prevalent human viruses detected by PCR in sewage and shellfish.

RV positivity in sludge and wastewater analyzed is in agreement with those obtained by Kittigul et al. (2005) which detected RV in 10 (25%) from 40 samples of sewage water analyzed.

HAV was detected in 25% of all analyzed samples. Other reports of HAV incidence on environmental samples are already described by Formiga-Cruz et al. (2005) with 20% of positivity in sewage samples and Villar et al. (2007) with 32% in raw and treated sewage samples.

Samples positive for HAV or PV or RV were also positive for human AdV. This observation is in agreement with previous studies (Pina et al. 1998; Formiga-Cruz et al. 2005) and also suggests that the detection of RNA viruses was not inhibited by natural levels of adenoviruses in the sewage samples studied.

ICC-PCR

For sewage and wastewater the 1:2 was selected as a non cytotoxic dilution to be used in MA104, A549 and Vero cells when ICC-PCR and IFA assays were applied. For FRHK-4 cells the 1:2 dilution was also selected for wastewater and 1:16 for sewage.

ICC-PCR results are described in Table 2. The positive sludge samples by ICC-PCR for HAV (16.7%) were different from the positive samples by nested RT-PCR (25%), but for wastewater 2 of 3 samples previous positive by nested RT-PCR were positive by ICC-PCR, showing that not all positive samples had viable HAV particles. HAV results corroborate with previous studies that indicate a low endemicity of HAV in–southern Brazil (Saback et al. 2001).

Interestingly,–PV has shown a high positivity by ICC-PCR (91.7%) when compared with RT-PCR reaction (66.7%), proving that the test can improve the sensitivity of the detection.

In this study, the high prevalence of viable PV particles found on sewage samples, can be probably derived from vaccine-derived PV strains, because this vaccination is part of the regular vaccination program in Brazil for all children under 5 years.

For RV from 33.3% genome positive sludge samples, 25% showed to be viable by ICC-PCR, but from 41.6% genome positive in wastewater, none of them showed to be viable by ICC-PCR. These results suggest a low infectivity of RV in wastewater.

Table 2 Detection of enteric viruses in sewage sludge and treated wastewater samples by ICC-PCR.

Sampling (month/year)	**AdV**		**HAV**		**PV**	**RV**	
	Sewage Sludge	Treated Wastewater	Sewage Sludge	Treated Wastewater	Sewage Sludge	Sewage Sludge	Treated Wastewater
06/2007	+	-	-	-	+	-	-
07/2007	+	-	-	-	+	-	-
08/2007	+	+	-	-	+	-	-
09/2007	+	-	-	-	+	+	-
10/2007	+	+	-	-	+	-	-
11/2007	+	+	-	+	+	-	-
12/2007	+	+	-	-	-	-	-
01/2008	+	+	+	+	+	+	-
02/2008	+	-	-	-	+	-	-
03/2008	+	+	-	-	+	+	-
04/2008	+	+	+	-	+	-	-
05/2008	+	+	-	-	+	-	-

+: positive sample. -: negative sample.

IFA for AdV quantification in sewage sludge

To confirm AdV viability showed by ICC-PCR, all sludge samples were tested by IFA, using A549 cells. This assay was also applied to quantify the virus particles in FFU/ml. As shown in Figure 1, the number of virus particles ranged from 70 FFU/ml (April, 2008) to 300 FFU/ml (August, 2007 and February, 2008).

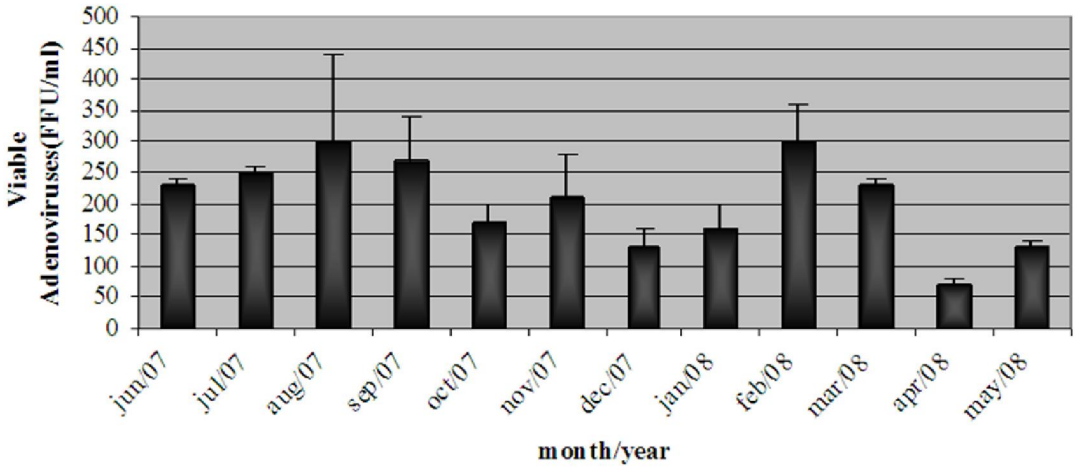

Figure 1. Quantification of viable AdV in sewage sludge samples by IFA.

Immunofluorescence staining is a very useful test for the detection of viral viability in cell culture and considered more reliable than the plaque assay (Birch et al. 1983). Regulation laws in Brazil, recommend that maximum concentration of enteric viruses that can be found in lots of sewage sludge should be lower than 0.25 PFU or FFU/g of total solids. Based on this information, we can infer that all field samples analyzed presented AdV contamination above the maximum allowed concentration to be used in agriculture.

The virus detection by molecular techniques, without cell culture, indicates only the presence of viral genome and does not provide any information on infectivity, which is directly related to the human health risk. With the AdV in sewage sludge, it was found that all field samples that had been submitted to the reaction of nested PCR were also positive by ICC-PCR and IFA, indicating the presence of viable viral particles in all samples analyzed. In wastewater samples only one sample positive by nested PCR was negative by ICC-PCR.

Quantification of HAV RNA and AdV DNA by Taqman RT-PCR

The results of quantification of viable viral genomes for quantitative RT–PCR PCR are shown in Table 3 and Figure 2.

Table 3 Number of genome copies/ml of HAV measured by real time RT-PCR in sewage sludge and treated wastewater samples.

Sampling (month/year)	Sewage Sludge (genome copies/ml)*	Treated Wastewater (genome copies/ml)*
06/2007	NT	NT
07/2007	NT	NT
08/2007	NT	NT
09/2007	NT	NT
10/2007	NT	$4.2x10^2$
11/2007	NT	$3.4x10^2$
12/2007	NT	NT
01/2008	$5.4x10^2$	$4.4x10^2$
02/2008	NT	NT
03/2008	NT	NT
04/2008	$3.1x10^2$	NT
05/2008	NT	NT

* Mean for duplicates with replies. NT: not tested

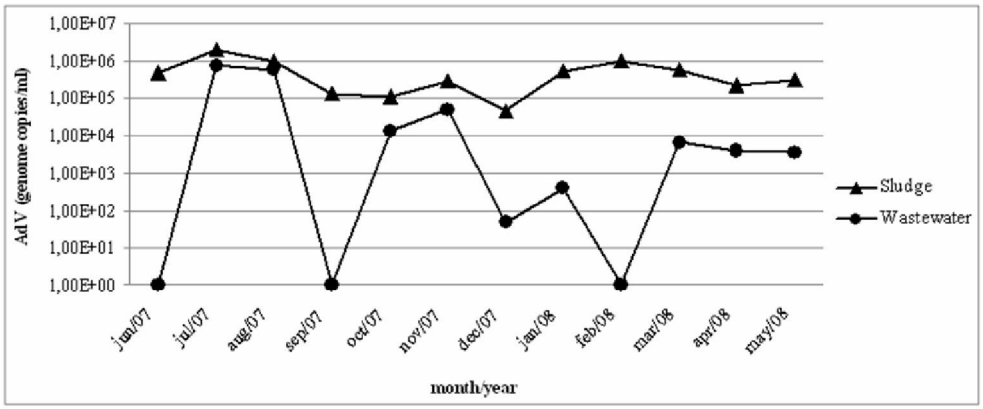

Figure 2. Quantification of AdV genomes in sewage sludge and treated wastewater samples by real time PCR.

The results found in this study by real time PCR were similar to the previously reported by Carducci et al. (2008) that showed a constant presence of AdV in sewage samples. Concerning HAV, it was observed that the number of genome copies/ml is markedly lower than that observed for AdV. These results corroborate with those obtained by Villar et al. (2007) where the number of genome copies/ml ranged from 1.7 $x10^2$ to 3.8 $x10^2$ in samples derived from treated sewage.

CONCLUSION

This study has shown the high prevalence of enteric viruses in treated activated sludges and treated wastewater effluent, applying molecular methods, integrated cell culture-molecular methods and immunofluorescence.

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Capítulo 4

VICTORIA, M.; **RIGOTTO, C.**; MORESCO, V.; CORRÊA, A.A.; KOLESNIKOVAS, C.K.M.; LEITE, J.P.; MIAGOSTOVICH, M.P.; BARARDI, C.R.M. Assessment of norovirus contamination in environmental samples from Florianópolis City, Southern Brazil. **Journal of Applied Microbiology**. Published Online: Jan 4 2010 3:22PM DOI:10.1111/j.1365-2672.2009.04646.x Fator de Impacto: 2,028.

Capítulo 5

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ASSESSMENT OF ADENOVIRUS, HEPATITIS A VIRUS AND ROTAVIRUS CONTAMINATION IN ENVIRONMENTAL SAMPLES IN FLORIANOPOLIS, SOUTH BRAZIL

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ABSTRACT

The importance of water in the transmission of Hepatitis A viruses (HAV), Rotavirus A (RV-A) and Human Adenovirus (HAdV) and their potential health risks are widely recognized. The aim of the present study was to assess the presence of these viruses on environmental samples from Florianopolis Island, Southern region of Brazil. Water samples from various sources and Pacific oysters (*Crassostrea gigas*) of two growing-areas in the island were analyzed. Enzymatic amplification (nested PCR and RT-PCR), quantification of HAdV genome copies and infection assay by integrated cell culture PCR (ICC-PCR) over a one-year period (June 2007 – May 2008) were used to this assessment. From a total of 84 water samples there were 54 (64.2%) positive for HAdV, 16 (19%) for RV-A and 7 (8.3%) for HAV. Viability assays in water samples showed non-positive samples for HAV, though infectious viruses were confirmed for RV-A, 2 of 16 (12.5%) and for HAdV, 48 of 54 (88.8%). From 24 oyster samples analyzed by nested PCR or RT-PCR, 87.5% were positive for HAdV, 8.3% for RV-A and none for HAV. Quantitative PCR in oysters samples showed means loads of 9.1×10^4 gc/g (oyster farm south) and 1.5×10^5 gc/g (oyster farm north) and in waters: 3.0×10^6 gc/L (lagoon), 8.1×10^6 gc/L (lagoon urban runoff), 1.9×10^7 gc/L (chlorinated drinking water), 1.1×10^7 gc/L (seawater south), 6.3×10^6 gc/L (seawater north), 9.7×10^6 gc/L (untreated drinking water) and 1.1×10^7 gc/L (polluted creek). The high loads of HAdV in the environment suggest the relevance of evaluating these viruses as positive indicators of viral contamination of water.

Key words: Environmental samples, HAV, Adenovirus, Rotavirus A, ICC-PCR, qPCR, PCR.

1. INTRODUCTION

In highly populated coastal areas, large amounts of treated and sometimes untreated human wastewater is discharged into environmental waters. In recent years, many researchers have demonstrated that the presence of human enteric viruses in various sources of water samples, such as raw sewage, treated sewage, river water, seawater and tap water, may affect the water quality and human health (Formiga-Cruz et al., 2005; Haramoto et al., 2005; 2007). The general demand for high-quality water has increased the pressure on environmental and public health policies to ensure the microbiological safety of water. To reduce human health risk from waterborne and water-related illness, water quality standards are established by the WHO (World Health Organization) and are adopted by most nations worldwide. Diseases caused by the consumption of bivalve mollusk shellfish containing pathogenic viruses from human origin are reported, particularly related to raw oysters. Human health problems associated with shellfish consumption are well described and contaminating viruses have been linked to nearly all episodes of gastroenteritis as well as outbreaks of illness related to consumption of contaminated shellfish (Le Guyader et al., 2008).

Bacterial indicators of fecal contamination were established as safety standards to assess water quality and the sanitary quality of shellfish. However, it has been clearly established that bacterial standards do not always reveal the presence of viruses in these samples (Formiga-Cruz et al., 2005). Moreover, outbreaks of viral diseases have occurred as a result of the consumption of water and mollusks with accepted values of coliform standards (Brooks et al., 2005; Le Guyader et al., 2008). On the other hand, improvements in molecular techniques have been conducted extensively to detect enteric viruses from environmental samples. More recently, polymerase chain reaction (PCR) method has become a major tool for virus detection, and different types of viruses have been isolated from water samples and mollusks, including fastidious viruses (Ko et al., 2005). PCR can also contribute to epidemiological studies because it is capable of differentiating specific viruses and also genotypes of the same virus through the use of specific primers. Disadvantages of conventional PCR methods include the fact that they do not differentiate between viable and nonviable viruses, or between whole virus, full length DNA and partial-length DNA. In this way, quantitative PCR (qPCR) is a powerful and increasingly popular technique for rapidly detecting and quantifying pathogenic microorganisms in clinical and environmental samples (Ko et al., 2005). By combining conventional cell culture methods and PCR amplification to a cell culture-PCR (ICC-PCR) assay, a method was obtained that combined selective

enumeration of infectious viruses with rapid detection. Such ICC-PCR assays have been successfully utilized for the detection of several other enteric viruses in environmental samples (Reynolds et al., 2004; Ko et al., 2005; Rutjes et al., 2009).

Since only few virus particles are present in water samples, virus detection always requires the prior concentration of the water sample such as adsorption and subsequent elution from electronegative or electropositive membranes. Recently, there has been much attention given to emerging enteric viruses because of their low infectious dose, survival in water, and considerable health impacts (from diarrhea to death). Human Adenovirus (HAdV), Hepatitis A virus (HAV) and Rotavirus A (RV-A) are excreted in the feces of infected individuals and may be dispersed into environmental waters. The stability of those viruses in environmental water and their resistance to water treatment may facilitate transmission to humans. In Brazil those viruses are responsible for a great number of cases such as in developing countries. Nevertheless, few studies have been conducted in the southern region of the country.

The State of Santa Catarina is a major producer of mollusks in Brazil due to excellent geographical conditions for marine organism cultures, especially bivalve mollusks such as the oyster species *Crassostrea gigas* (Santos, 2001). Moreover, Florianopolis city (27°S, 48°W), composed of one main island, a continental part and surrounding small islands, has a very important touristic trade due to the natural landscape including more than 40 beaches. The increasing pollution of the coastal waters of Santa Catarina State requires monitoring of cultured bivalve mollusks to ensure health safety standards. In this study, the distribution of HAdV, HAV and RV- A was evaluated in water samples and shellfish from Florianopolis for a one-year period (June 2007 – May 2008), by enzymatic amplification (conventional PCR), quantification by real time PCR and viability by integrated cell culture PCR (ICC-PCR).

2. MATERIALS AND METHODS

2.1. Environmental water and oyster samples

A total of 84 water samples (2 L each) were collected monthly over a period between June 2007 to May 2008 in seven different collection sites (Figure 1) in Florianopolis, as follows: seawater was collected from two different sites of oyster farms, one in the northern (seawater north) and another in the southern (seawater south) of the island; lagoon brackish water was collected from an important recreational area; urban wastewater thrown in the former lagoon; two drinking waters (with and without chlorination) and the last site located in

a polluted creek in a suburban area of the city. From June 2007 to May 2008, Pacific oysters (*Crassostrea gigas*) samples were collected monthly from two oysters farms, one in the northern region (oyster farm north) and another in the southern region of the island (oyster farm south). Each sample consisted of 12 oysters that were dissected to extract the digestive tissue for virological analysis. All water and oyster samples were kept at 4°C in sterile containers and processed within 24 h of collection.

2.2 Viruses concentration method from water samples

Viruses concentration in water was performed by adsorption in an electronegative membrane and subsequent elution as described by Katayama et al. (2002) with minor modifications. This adsorption-elution method was already evaluated by Victoria et al. (2009) for the detection of enteric viruses in environmental waters tested in the present study.

Treated drinking water was dechlorinated with 80 mg/L of sodium thiosulfate before filtration step. Water samples were filtered into an HA (mixed cellulose esters) negatively charged membrane (Nihon Millipore®, Tokyo, Japan) with a pore size of 0.45 µm and 142 mm diameter that was placed into a vacuum pump. Viruses were then adsorbed in presence of 25 mM $MgCl_2$ (except for seawater). The membrane was rinsed with 350 mL of H_2SO_4 (0.5 mM, pH 3.0) to elude cations and subsequently treated for 10 min with 15 mL of NaOH (1.0 mM, pH 10.5) to allow the elution of viruses. The filtrate was neutralized with 50 µl of 50 mM H_2SO_4 and 100× TE buffer (pH 8.0) and then immediately ultrafiltered with a Centriprep Concentrator 50® system (Nihon Millipore®, Tokyo, Japan) at 1500×g for 10 min at 4°C to obtain a final volume of 2 mL. Four hundred microliters of 2 mL was further used for nucleic acid extraction. For some samples, an additional step of filtration with AP20 membrane was included in the procedure when the concentration was carried out in order to eliminate debris.

2.3. Virus concentration method from oyster samples

After sampling, oysters' shells were opened at the hinge with a sterile oyster knife for digestive tissues dissection. For the recovery of viral particles, the method described by Lewis and Metcalf (1988) with minor modifications was applied. Briefly, the solids-adsorbed viruses were eluted from 2 g of hepatopancreas homogenate (corresponding to tissues derived from 12 dissected oysters) by resuspension in 10 mL of tryptose phosphate broth (TPB) dissolved in 0.05 M glycine pH 9.0. The homogenate was purified with an equal volume of chloroform-butanol (1:1) and centrifuged at 13,500 g at 4°C for 15 min. Viruses in the supernatant were precipitated with 12% polyethylene glycol (PEG), dissolved in 0.75 M NaCl for 2 h at 4°C

and centrifuged at 13,500 g and 4°C for 20 min. Each pellet was resuspended in 3 mL of ultrapure water, and clarified twice with 30% of chloroform.

2.4. Cell lines and viruses

For virus viability assays from environmental water, HAV-cytophatic strain HAF 203, Human AdV 5 (HAdV5) (genogroup C, serotype 5) and Simian RV SA11 (group A, serotype G3), were respectively propagated in a continuous line of fetal FRHK-4 cells (rhesus kidney-derived cells), Hep-2 cells (human larynx carcinoma) and MA104 cells (a continuous line of fetal rhesus kidney cells).

2.5. Cytotoxicity assays

Cytotoxic assays were performed using 2-fold serial dilution (1:2 to 1:32) in serum-free Minimum Essential Medium Eagle (MEM) of unseeded autoclaved water samples. Plates were incubated and observed for cytotoxic effects after 48h for MA104 and 72h for Hep-2 cells; and up to a week for FRhK-4 cells. Each observation was compared with negative controls containing only normal cell monolayer and medium. After period of incubation, the cellular integrity was confirmed by removing the medium by suction and staining with 250 µl of naphthalene black (Sigma - Aldrich, USA) at 0.1% in 5% acetic acid pH 2.3. After 30 min incubation, the stain was removed by suction and the cells were air dried and observed under an inverted microscope. The cells were analyzed to establish the first non-cytotoxic dilution to be used at ICC-PCR.

2.6. Integrated cell culture PCR (ICC-PCR)

ICC-PCR was performed as previously described by Rigotto et al. (2005) and applied to check the viruses' viability in environmental water samples previously positive for conventional PCR. FRHK-4, Hep-2 and MA104 cells monolayers were prepared in 24-well microplates. The water samples and the positive controls (infected cell supernatant of AdV5 or HAV-cytophatic strain HAF 203 or Simian RV SA11) were diluted in maintenance medium. Inocula were then allowed to adsorb to PBS (150 mM NaCl, 1.5 mM KH_2PO, 20 mM Na_2HPO_4, 27 mM KCl, pH 7.2)-washed cell-layer for 90 minutes at 37°C with rocking every 15 minutes, and non-adsorbed virus were removed from cell monolayers by washing with PBS for three times. After incubation as described above, plates were subjected to three cycles of freeze/thaw for cell lyses and virus releasing. The supernatant was recovered and 0.5 ml was used for viral genome isolation and PCR detection.

2.7. Nucleic acid extraction

Viral nucleic acids were extracted by a procedure described by Boom et al. (1990). This procedure uses guanidinium thiocyanate and adsorption of the nucleic acids to silica particles. To reduce the presence of inhibitors of the PCR reactions, a 10-fold dilution of RNA/DNA of each sample was carried out.

2.8. Enzymatic amplification – qualitative and quantitative PCR

2.8.1. Reverse transcription

The reverse transcription reaction for RNA viruses was performed by using random primer (hexamer $pd(N)_6$ - 50 A_{260} units - Amersham Biosciences, Buckinghamshire, UK) and SuperScript™ III Reverse Transcriptase (Invitrogen™, CA, USA) following manufacturer's recommendations.

2.8.2. Qualitative PCR

HAdV DNA was detected in samples by using the oligonucleotide primers pair hexAA 1885 /hexAA 1913 and nexAA 1893/nexAA 1905 described by Allard et al. (1992). The expected size of the PCR product was 300 basepair (bp) and 142 bp for nested PCR.

HAV RNA was detected by nested RT-PCR, using the oligonucleotide primers pair F6 (+) and F7 (-), which amplifies a 392 bp fragment, suitable to amplify all HAV genotypes ('universal primers'). Internal primers were F8 (+) and F9 (-), which amplifies a 247 bp fragment (De Paula et al., 2004).

RV-A RNA was detected by RT-PCR, using the oligonucleotide primers pair VP6-F (+) and VP6-R (-), which amplifies a 379 bp fragment of the VP6 gene (Iturriza-Gómara et al., 2002).

2.8.2. Quantitative PCR (qPCR)

Oysters samples previously positive by PCR and water samples previously positive by PCR and ICC-PCR were quantified in duplicate by qPCR using the ABI 7500 Real-Time PCR System® (Applied Biosystems, CA, USA) following the manufacturer's instructions. For the specific detection and quantification of HAdV genomes, 10 µl of the 10-fold and 100-fold dilutions of every DNA extraction were also assayed. These dilutions were made to avoid amplification inhibition due to this assay's high sensitivity to inhibitors.

The reaction for HAdV consisted of 10 µl of a DNA sample or 10 µl of a quantified HAdV41 cloned in pBR322 (kindly donated by R. Girones, Barcelona University) in a 25 µl reaction mixture with the PCR Master Mix (Applied Biosystems). HAdV genomes were quantified with 0.9 µM the primers AdF and AdR and 0.225 µM of the AdP1 probe described by Hernroth et al. (2002). All samples were run in quadruplicate, and positive and negative controls were included. The amount of DNA was defined as the average of the quadruplicate data obtained.

2.9. Quality control

To avoid the number of false positives resulting from carryover contamination of amplified virus particles or viral nucleic acid, separate areas and equipment were used for each stage of the process. Negative controls (non-spiked autoclaved distilled water) and positive controls (virus suspensions) were included with each set of test samples and taken through nucleic acid extraction and enzymatic amplification assays. Additional blank controls (containing the same reaction mixture except for the nucleic acid template) were incorporated with all PCR assays.

3. RESULTS

3.1. Water samples

Water samples were analyzed over one year in order to detect HAdV, HAV and RV by nested PCR, nested RT-PCR, and RT-PCR, respectively. From a total of 84 water samples there were 54 (64.2%) positive for HAdV with the highest incidence (75.0%) in lagoon water and polluted creek and lowest incidence (33.3%) in non-treated drinking water. RV-A showed 16 (19.0%) positive waters with highest value of 58.0% in polluted creek. HAV achieved a total of 7 (8.3%) positive samples with 16.6% in lagoon, seawater north and non-treated drinking water (Table 1).

Seasonal distribution of these viruses is shown in Figure 2. HAV and RV-A showed a similar seasonal pattern showing higher concentrations in winter months (July and August 2007) and in summer the (December 2007 and January 2008) (t-test, $P < 0.05$). The highest incidence of RV-A (42.8 %) had occurred in December 2007 and January 2008. For HAV the highest incidence was in July and October 2007 (28.6 %). On the other hand, for HAdV there was a widespread distribution in the environment with the highest incidence in July and

November 2007 (100%) and absence only in September 2007. Positive samples by qualitative nested PCR were further analyzed by ICC-PCR to verify the virus viability in cell culture.

For all environmental waters, the 1:2 dilutions were selected as a non-cytotoxic condition to be used in MA104, Hep-2 and FRhk-4 cells when ICC-PCR was applied. ICC-PCR was performed to verify viruses' infectivity from the previously positive water samples by qualitative PCR. ICC-PCR results are described in Table 1. From the 7 previously positive samples for HAV by RT-nested PCR, none of them were positive by ICC-PCR. However, infectious viruses were confirmed for HAdV, 48 of 54 (88.8%) and for RV, 2 of 16 (12.5%).

HAdV previously positive samples by qualitative nested PCR were also analyzed by qPCR. The concentration of virus detected is expressed as genomic copies (gc) per L, and the results are shown in Figure 3. The levels of HAdV were high in all samples (mean of sites: 9.8 x 10^6 gc/L), with means loads of 3.0 x 10^6 gc/L (lagoon), 8.1 x 10^6 gc/L (lagoon urban runoff), 1.9 x 10^7 gc/L (chlorinated drinking water), 1.1 x 10^7 gc/L (seawater south), 6.3 x 10^6 gc/L (seawater north), 9.7 x 10^6 gc/L (untreated drinking water) e 1.1 x 10^7 gc/L (polluted creek).

3.2. Oyster samples

From the 24 oysters samples analyzed by conventional PCR (Table 2), none of them had amplification for HAV, 2 of 24 (8.3%) were positive for RV-A, one from oyster farm north (June 2007) and the other from oyster farm south (December 2007). For HAdV, at north site 10 of 12 were positive (exceptions were august 2007 and November 2007) and at south site 11 of 12 were positive (except July 2007). Positive samples for HAdV were quantified by real time PCR.

Quantitative PCR results for oysters samples are summarized at Table 2. The levels of HAdV in oyster farm south and north are high (average of 9.1 x 10^4 GC/g and 1.91 x 10^5 GC/g, respectively). All the negative controls performed in these assays proved negative.

4. DISCUSSION

The present study describes the distribution, viability and quantification of HAdV, HAV and RV-A in water and oysters samples in Florianopolis Island, southern region of Brazil. For water samples an adsorption–elution method with an HA negatively charged membrane methodology was used to concentrate those viruses. This method has been used in

other studies (Haramoto et al., 2005; 2007), including some performed in Brazil (De Paula et al., 2007; Miagostovich et al., 2008; Ferreira et al., 2009; Victoria et al., 2009) and has been successful for detecting enteric viruses from environmental samples. It is based on the use of inorganic solution for viruses' adsorption and elution, avoiding the use of organic solutions as beef extracts which is well recognized as an important inhibitor of enzymatic amplification reactions. Even though a pre-dilution of the purified nucleic acids prior to the RT-PCR reaction was performed in this study to ensure specificity of detection, eliminate any false-positive results, and increase the amplification signal as previously suggested by Brooks et al. (2005). Here, only six samples presented inhibition revealed by the samples that became positive after dilutions in RT-PCR and RT-nested PCR reactions being two for RV-A and four for HAV (data no shown).

The occurrence of those viruses in aquatic environments, showed a high prevalence of HAdV in water and oyster samples. The primers used for HAdV were designed to detect all human adenovirus types, and therefore target a conserved region of the hexon gene of human HAdV (Hernroth et al., 2002). During the survey period, HAdV were detected in all seven kinds of water and in 2 oyster samples (oyster farms north and south), thus showing a widespread in the environmental samples, which is in accordance with previous studies that have also investigated for the presence of HAdV in oyster samples in the same geographic region (Rigotto et al., 2005).

RV-A detection in oyster was also an important finding mainly due to contamination of shellfish-growing in water has been a cause of gastroenteritis outbreaks and it has been well documented since the late 19th and early 20th centuries (Le Guyader et al., 2008). The high prevalence of HAdV in water and oyster samples indicates that there was no occurrence of seasonality. These results are consistent with reports from Formiga-Cruz et al. (2005) which found that HAdV are excreted throughout the year in higher numbers than enteroviruses and HAV. In temperate climate regions the prevalence of HAdV is higher than other regions. Although in tropical regions, as in Brazil, HAdV in water have been detected during all months of the year (Horwitz, 1996). Overall, the prevalence of HAdV in this study was 64.2%, 54 out of the 84 samples. It is well known that virus detection by molecular techniques, without cell culture, indicates only the presence of viral genome and does not provide any information on infectivity, which is directly related to the human health risk. Infections assays in this study (ICC-PCR) have confirmed the HAdV viability in 88.8% (48/54), of the cases same result was achieved with qPCR. Despite carrying out DNA dilutions of HAdV, it was not detected in 6 samples among the 54 previously tested positive

by nested PCR, this may be due to the presence of high concentration of inhibitors in these samples.

The mean loads of HAdV in this study ranged from 3.0 x 10^6 to 1.9x10^7 gc/L in water samples and from 9.1 x 10^4 to 1.5 x 10^5 gc/g in oysters samples. Less than 1 log difference in viral load was found between all water samples and also between the two oyster farms. This viral load could represent a risk of infections in the population if efficient drinking-water treatment is not applied and if population uses the surface water as a recreational area. Previous work has shown that there is a poor correlation between levels of viruses and the current recreational water indicators (Wong et al., 2009). Examination for the presence of viruses in recreational waters, surface waters, drinking water and oysters is currently not required by legislation in Brazil, but the monitoring of human enteric viruses in water and shellfish samples by using molecular detection has been performed in many laboratories around the world (Katayama et al., 2002; Boxman et al., 2006).

Viral loads results from this study corroborate with those described by Formiga-Cruz et al. (2005) which showed that the HAdV are the most prevalent human viruses detected by PCR in sewage and shellfish. HAdV have been described as stable in the environment and highly resistant to UV disinfection (Thompson et al., 2003) and have been found previously to be prevalent in surface water (Hundesa et al., 2006).

The results for HAdV reported here, support the hypothesis that these viruses are a suitable index for human viral environment contamination. One of the reasons is the high number of samples which were positive for HAdV but negative for RV-A and HAV. RV-A was more prevalent (58.0%) in polluted creek, similar result achieved for HAdV so that suggests a possible discharge of non-treated sewage in this creek. RV-A is shed in extremely high numbers (up to 10^{10}/g) from the feces of infected individuals and can persist in the environment for extended periods of time (Hamza et al., 2009). Furthermore, RV-A was confirmed viable (28.6%) in polluted creek by ICC-PCR, although it has not been found in other samples. This result is also in agreement with Rutjes et al. (2009), which in 55.0% of the samples, rotavirus genomes were 1,000 to 10,000 times (3 $\log_{10}$–4 $\log_{10}$) more abundantly present than infectious rotavirus particles by ICC-PCR.

HAV was detected in lagoon water, lagoon urban runoff, and seawater north and non-treated drinking water. However, applying the ICC-PCR assay, we have not found any positive (viable) HAV. This result suggests that the serotypes present in positive samples might be from fastidious HAV strains. Moreover, no samples have been found positive for HAV in oysters. HAV results corroborate with previous studies that indicate a low endemicity

of HAV in southern Brazil (Saback et al. 2001). Other reports of HAV in environmental samples have also shown a low incidence: 20% in sewage samples (Formiga-Cruz et al., 2005), and 32% of HAV in raw and treated sewage in Brazil (Villar et al., 2007).

Samples positive for HAV or RV-A were also positive for human HAdV. This observation is in agreement with previous studies (Formiga-Cruz et al., 2005) and also suggests that the detection of RNA viruses was not inhibited by natural levels of adenoviruses in the samples studied.

This study with an environmental approach provides data concerning the prevalence, viability and quantification of enteric viruses in environmental waters and oysters in Florianopolis Island, southern region of Brazil in order to better understand the distribution of these viruses in this region of the country.

CONCLUSIONS

Florianopolis Island has been adversely impacted by human fecal pollution and the levels of pathogens detected in this study indicate that they might pose a risk to population in contact with the environmental waters searched. It is therefore particularly important to develop water quality indicators for contamination with human sewage to address viral risks.

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Table 1: Detection of Rotavirus- A (RV-A), hepatitis A virus (HAV) and Human Adenovirus (HAdV) in environmental water samples by conventional polymerase chain reaction (PCR) methods and integrated cell culture (ICC)-PCR, analyzed over a period from June 2007 to May 2008.

Water sample	**RV-A**	**HAV**				**HAdV**
	% (number of positive/tested)					
	RT-PCR	**ICC-PCR**	**RT-Nested PCR**	**ICC-PCR**	**Nested-PCR**	**ICC-PCR**
Lagoon water	16.6 (2/12)	- (0/2)	16.6 (2/12)	- (0/2)	75.0 (9/12)	88.8 (8/9)
Lagoon urban runoff	8.3 (1/12)	- (0/1)	8.3 (1/12)	- (0/1)	66.6 (8/12)	100.0 (8/8)
Polluted Creek	58.3 (7/12)	28.6 (2/7)	0 (0/12)	-	75.0 (9/12)	100.0 (8/8)
Seawater North	25.0 (3/12)	- (0/3)	16.6 (2/12)	(0/2)	66.6 (8/12)	100.0 (7/7)
Seawater South	25.0 (3/12)	- (0/3)	0 (0/12)	-	66.6 (8/12)	85.7 (6/7)
Chlorinated drinking water	(0/12)	-	0 (0/12)	-	66.6 (8/12)	50.0 (4/8)
Non-treated drinking water	(0/12)	-	16.6 (2/12)	(0/2)	33.3 (4/12)	100.0 (4/4)
Total % Positive	**19.0 (16/84)**	**12.5 (2/16)**	**8.3 (7/84)**	**(0/7)**	**64.2 (54/84)**	**88.8 (48/54)**

[a] ICC-PCR samples analyzed were selected from previously found to be positive by nested PCR or RT-PCR. Results expressed in %.

Table 2: Detection and quantification of Rotavirus A (RV-A), hepatitis A virus (HAV) and human Adenovirus (HAdV) in oysters samples by conventional polymerase chain reaction (PCR) methods and quantitative PCR (qPCR) analyzed over a period from June 2007 to May 2008.

Origin of Oyster samples	**RV-A**		**HAV**		**HAdV**	
	RT-PCR [a] % (positive/tested)	**qRT-PCR Mean (min-max)**	**RT-Nested PCR [a]** % (positive/tested)	**qRT-PCR Mean (min-max)**	**Nested-PCR [a]** % (positive/tested)	**qPCR Mean (min-max)**
Farm South	8.3 (1/12)	NT [c]	(0/12)	NT [c]	91.6 (11/12)	9.1×10^4 1.3×10^4 – 1.9×10^5
Farm North	8.3 (1/12)	NT [c]	(0/12)	NT [c]	83.3 (10/12)	1.5×10^5 3.1×10^4 – 6.0×10^5
Total % Positive	8.3 (2/24)		(0/24)		87.5 (21/24)	100

[a] Results expressed as %. [b] Results obtained in quantitative PCR from previously found to be positive by nested PCR or RT-PCR. Results expressed in GC/g. [c] NT: not tested.

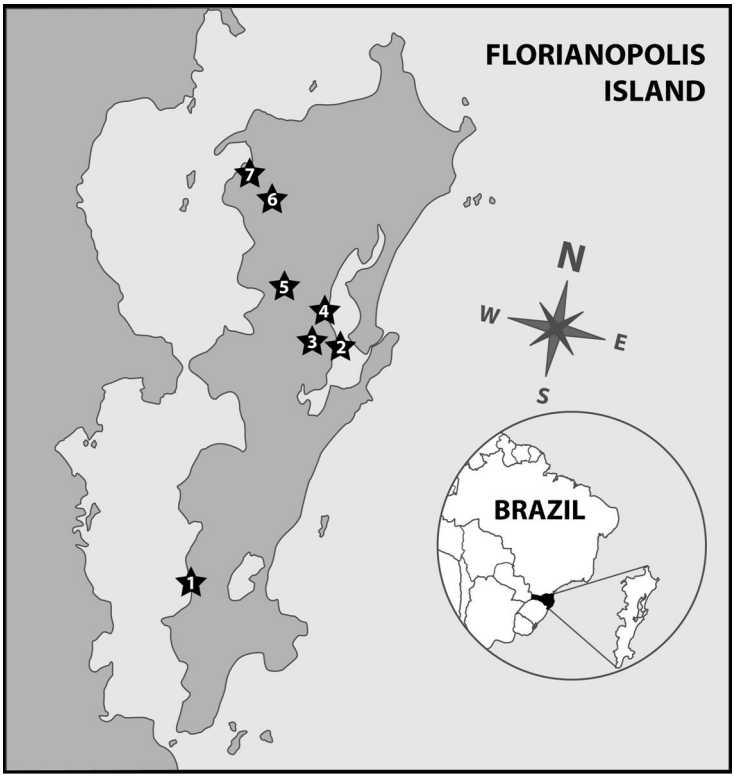

Figure 1: Map of sampling area. Numbers indicate the collection sites in Florianopolis Island, Southern Brazil. 1- seawater and oyster farm south, 2- recreational lagoon water, 3- chlorinated drinking water, 4- lagoon urban runoff, 5- polluted creek, 6- untreated drinking water, 7- seawater and oyster farm north.

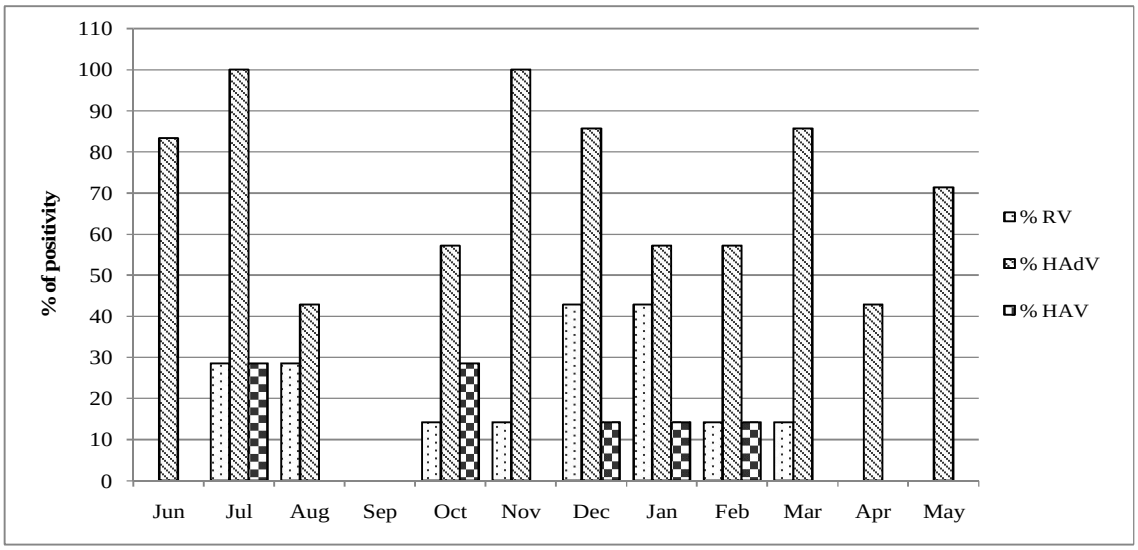

Figure 2: Seasonal distribution of Rotavirus A (RV-A), Human Adenovirus (HAdV) and Hepatitis A Virus (HAV) in environmental waters over a period from June 2007 to May 2008. Values in percentage of positivity.

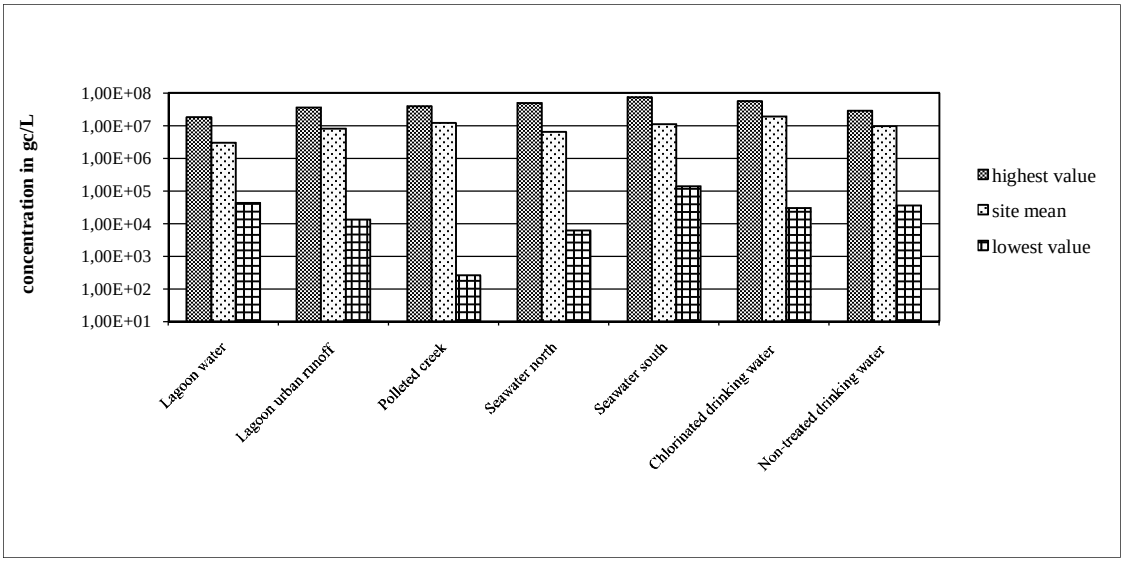

Figure 3: Concentration of Human Adenovirus (HAdV) in water samples by quantitative PCR analyzed over a period from June 2007 to May 2008. Values in gc/L.

Capítulo 6

RIGOTTO, C.; HANLEY, K.; ROCHELLE, P.A.; DE LEON, R.; BARARDI, C.R.M.; YATES, M.V. Survival of adenoviruses serotypes 41 and 2 in surfaces and ground waters measured by a plaque assay. *Artigo em fase de finalização*.

SURVIVAL OF ADENOVIRUSES TYPE 2 AND 41 IN SURFACE AND GROUND WATERS MEASURED BY A PLAQUE ASSA

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Abstract

In order to manage artificial recharge systems, it is necessary to understand the decay process of microorganisms within aquifers so that requirements regarding storage times and treatment strategies of ground and surface waters can be developed and modeled to improve water management practices. This study was designed to investigate the survival of representative adenoviruses in surface and ground waters using a cell culture plaque assay with human lung carcinoma cells (A549) to enumerate surviving viruses. Known concentrations of Adenovirus types 2 (Ad2) and 41 (Ad41) were seeded into 50 mL of 3 different surface waters and ground waters, and kept at 10 and 19°C. There was a small amount of reduction in infectious virus (Ad2) counts in surface water after 23 weeks at 10°C, with losses ranging from approximately 0.5 to 0.75 $\log_{10}$ and in ground water from less than 0.5 to 0.5 $\log_{10}$. At 19°C, Ad2 virus losses were somewhat higher, ranging from approximately 1.0 to 1.3 $\log_{10}$ in surface water and from 0.8 to 1.1 $\log_{10}$ in ground water. The reductions in the infectious virus counts of Ad41 at 10°C were lower than those observed for Ad2, ranging from 0.02 to 0.12 $\log_{10}$ in surface water and from 0.11 to 0.25 $\log_{10}$ in ground water. The greatest reduction of Ad41 was at 19°C, with values ranging from approximately 0.9 to 1.2 $\log_{10}$ in surface water and from 0.9 to 1.1 $\log_{10}$ in ground water. These results demonstrate the relatively high environmental stability and long-term survival of these adenoviruses in environmental waters. This long-term survival should be considered in risk assessment models and drinking water management strategies.

Key words: Adenovirus, plaque assay, A549 cells, water, survival.

1. Introduction

Outbreaks of infectious diseases continue to be linked to consumption of drinking water in both developed and developing countries. It has been estimated that 30-90% of waterborne outbreaks in the world are related to viruses (Bosch et al., 2008).

Human adenoviruses (HAdVs) are double-stranded DNA nonenveloped viruses, with 52 serotypes organized in subgroups A to F; subgroup F is composed of the enteric adenoviruses most associated with diarrhea in children. Disease symptoms from adenovirus infection in humans also include respiratory illness, gastroenteritis, and conjunctivitis (Lewis et al., 2009). This group of viruses has been detected in different sources of waters, and although they are readily inactivated by conventional free chlorine disinfection, they are more resistant to UV disinfection (Yates et al., 2006) and UV combined with monochloramine, than enteroviruses and other pathogens of concern to the water industry (Sirikanchana et al., 2008).

Adenovirus resistance and the virus's long persistence in the environment have increased the interest in monitoring the presence of adenoviruses in water. Assessing the risk to public health from pathogens in drinking water requires knowledge of the type and concentration of pathogen, infectious dose, and survival characteristics in natural waters. A few virus survival studies have been conducted evaluating poliovirus 1, echovirus 1, and MS-2 coliphage in groundwater (Yates et al., 1985) and enteric adenovirus 40 and 41 in tap, sea, and waste water (Enriquez et al., 1995).

Cell culture is the most commonly used method for the isolation of infectious viruses from water. The study of enteric adenovirus in water has been hindered because they are so-called "fastidious" viruses: they may infect cells without producing visible cytopathic effects (CPE) and are generally more difficult to culture than other HAdV species (Cromeans et al., 2008). Many investigators use the G293 cell line to propagate HAdVs 40 and 41, though lengthy incubation is required usually for appearance of CPE and no plaque assay has been reported using G293 cells. Although Grabow et al. (1992) have reported a plaque assay for HAdV 41 in PFL/PRF/5 cells, other investigators have not reported application of this assay. In the same way, other investigators (Hashimoto et al., 1991) developed a plaque assay for HAdV 40 Sapporo strain in a lung carcinoma cell line (A549). However, other investigators did not find this assay possible with the HAdV 40 (Dugan strain) obtained from American Type Culture Collection (ATCC), (Mautner, 1999). In the absence of a standard plaque assay for the enteric adenoviruses, a most-probable-number (MPN) approach has typically been used for the quantitation of infectious HAdVs 40 and 41. Recently, however, Cromeans et al.

(2008) successfully developed a plaque assay in A549 cells using HAdV species F; Tak (HAdV 41) and Dugan (HAdV 40) strains from the CDC archives.

This study was designed to investigate the long term survival of representative adenoviruses (Ad2 and Ad41 Tak strain) in surface and ground waters using a cell culture plaque assay to enumerate surviving viruses.

2. Materials and methods

2.1. Source of water

Ground water sample 1 (GW1) was obtained from Los Angeles, CA; ground water sample 2 (GW2) from Riverside, CA; and ground water sample 3 (GW3) and the 3 surface waters samples (SW1, SW2 and SW3) were obtained from the Metropolitan Water District of Southern California, La Verne. All samples were kept in sterile containers and autoclaved before use.

2.2. Experimental procedure

Adenovirus types 2 and 41 were added into separate 50-mL samples of environmental waters contained in sterile polypropylene centrifuge tubes (Corning Life Sciences, Lowell, Massachusetts) to achieve a final concentration of $3.3 \times 10^3 - 1.3 \times 10^4$ plaque forming units per mL. The tubes were capped and incubated at 10 and 19°C. Subsamples (1 mL) were withdrawn at regular intervals. Chemical analysis of surface and ground waters were conducted using procedures from *Standard Methods for Examination of Water and Wastewater* (APHA, 2005).

2.3. Statistical analyses

All statistical analyses were performed using Microsoft Excel. The concentration of virus at time t (Nt) was divided by the initial concentration (N0). The log10 of the ratio (Nt/N0) was plotted as the y value versus time (days) as x. Linear regression analysis was used to determine the decay rate for each virus, using the general equation: $Y = mx + b$; where "m" represents the slope or the inactivation rate of the virus, "y" is log10 (Nt/N0), "x" is the time in days, and "b" is the y-intercept. The slope of the regression line was determined to be the decay rate for the virus in each survival experiment. The decay rates for each individual experimental replicate were averaged to get an overall inactivation rate for a specific virus within a water sample at each incubation temperature.

The decay rates for each replicate were averaged and the average value was used in the final ANOVA statistical analysis. One-way ANOVA analysis was performed to determine if there was a difference between the decay rates of the 3 samples of each water (SF1, SF2, SF3, GW1, GW2, GW3) for each virus at one temperature. In addition, one way ANOVA analysis was performed to determine if there was a difference between the decay rates for each virus using each temperature as a treatment variable.

In the comparison of the inactivation rates for each virus within each water sample at different temperatures, the null hypothesis H0 was that there is a difference between the survival of each virus in the different temperatures. When comparing the different temperatures, H0 = there is a difference between the different temperatures and the alternative hypothesis, Ha = there is no difference between the different temperatures.

2.4. Virus and cells stocks production

The lung carcinoma epithelial cells (A549) were obtained from ATCC (American Type Culture Collection) and maintained with Dulbecco's Modified Eagle Medium (DMEM, Gibco - Invitrogen Carlsbad, CA) supplemented with 10% FBS (Foundation, USA), 1 mM sodium pyruvate, 10 units/ml penicillin sodium and 10 µg/ml streptomycin. Monolayers were subdivided weekly at a split ratio of 1:7-1:10 until no more than passage 93. Adenovirus type 2 (Ad2) (Adenoid 6 strain) was obtained from ATCC and Adenovirus type 41 (Ad41) (Tak strain) was kindly donated by Dr. Theresa Cromeans from CDC archives. Viruses were propagated in A549 cells and cells debris from virus suspensions were extracted with an equal volume of chloroform by shaking vigorously for 1 min, followed by separation of the aqueous layer with a low-speed centrifugation (2500×g) for 15 min. The upper aqueous layer was removed, aliquoted and stored at −80°C until use.

2.5. Plaque Assay

The plaque assay was performed as described by Cromeans et al. (2008) with minor modifications. Briefly, A549 cells were seeded in 60 mm dishes at 3 x 10^5 cells/well incubated at 37°C with 5% CO_2 until 80% confluence. Water samples were serially diluted in Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma-Aldrich) and non-cytotoxic dilutions of 0.24 ml were inoculated in triplicate onto monolayers that had been pre-washed with DPBS. After 1h incubation at 37°C with rotation every 15 min., the inocula were removed and cells received an agar overlay prepared with DMEM at 2x concentration with additions to

achieve final 1×concentrations of 2% FBS, 0.1 mM sodium pyruvate, 10 units/ml penicillin G sodium, 10 µg/ml streptomycin sulfate, and 26 mM $MgCl_2$.

The DMEM 2X media was combined with 1% agarose (SeaKem ME, Lonza, USA) to prepare the overlay containing 0.5% agarose. Complete DMEM 2X and 1% agarose solutions were warmed to 44°C in a water bath before added to the monolayer.

After seven days of incubation for both viruses at 37°C with CO_2, macroscopic virus plaques were visualized by staining with crystal violet and enumerated.

2.6. Viral confirmation assays

Virus confirmation assays were performed in order to confirm that the produced plaques were from Adenovirus 2 and 41. Selected plaques before staining were removed from agar, dissolved in DPBS and used to inoculate fresh A549 cells. After the incubation period of 3 days, the mRNA of infectious viruses was extracted using a RNasy Qiagen kit (Qiagen, Inc, Valencia, California, USA), and Integrated Cell Culture RT-PCR (ICC/RT-PCR) was performed as described by Ko et al. (2005b) using fiber (Ad41) and hexon (Ad2) primers. Amplified fragments were visualized by standard agarose gel electrophoresis.

3. Results

3.1. Characteristics of surface and ground waters used for survival studies

The results of the cation, anion, and alkalinity measurements for each surface and ground waters are listed in Table 1.

3.2. Virus survival

ANOVA showed that there was not a significant difference (i.e., $p > 0.01$) between the decay rates of either of the viruses in the three different surface and ground waters (data not shown). Table 2 presents the decay rates of Ad2 and 41 obtained by the inactivation rates $[\text{Log}_{10} (N_t/N_0)/t]$ for the three water samples from each kind of water using plaque assay. The null hypothesis (H0 = there is a difference between the different temperatures) was accepted only when comparing 10 and 19°C for Ad41 in surface water ($p<0.01$).

Overall, the inactivation rate of Ad2 was higher than that of Ad41 at both temperatures (Table 2). There was a little reduction in infectious virus (Ad2) counts [Figure 1(c);(d)] in surface water after 161 days at 10°C, with losses ranging from approximately 0.5 to .75 $\log_{10}$ and in ground water from less than 0.5 to 0.5 $\log_{10}$. At 19°C, Ad2 virus losses were somewhat

higher, ranging from approximately 1.0 to 1.3 log_{10} in surface water and from 0.8 to 1.1 log_{10} in ground water. The reductions in the infectious virus counts of Ad41 [Figure 1 (a);(b)] at 10°C were lower than those observed for Ad2, ranging from 0.02 to 0.12 log_{10} in surface water and from 0.11 to 0.25 log_{10} in ground water. The greatest reduction of Ad41 was at 19°C, with values ranging from approximately 0.9 to 1.2 log_{10} in surface water and from 0.9 to 1.1 log_{10} in ground water.

3.3. Viral Confirmation Assays

Plaques from Ad2 and Ad41 were confirmed to be positive by ICC/RT-PCR for both viruses. Fiber primers design for human adenovirus from group F (Ad41) amplification were able to amplify Ad41 from tested plaques, moreover samples from Ad2 plaques tested with the same primers were confirmed negative. The negative result for Ad2 in Ad41 samples shows that there was not cross-contamination between samples.

4. Discussion

Although the role of water in transmission of adenoviruses is unclear, a few outbreaks of adenovirus infection have been associated with recreational water (Martone et al., 1980; Craun et al., 2003). Assessments of health risk posed by adenovirus in drinking water are hampered by a lack of information on the infectious dose of enterically transmitted adenovirus. In addition, a standard analytical technique based on virus viability needs to be developed and applied in surveillance, infection, survival and persistence of adenovirus in source waters.

Human Adenoviruses species F (Ad40 and 41) from clinical samples have been reported by numerous investigators as difficult to culture (de Jong et al., 1983; Mautner, 2007). Most investigators have employed the G293 cell line for isolation and propagation of species F of HAdV. However, some investigators have found other cell lines useful for propagation of these viruses, as PLC/PRF5 and Caco2. Although the A549 cell line has not been considered useful for HAdV 40 or 41 cultures, some investigators have propagated isolates of HAdV 40 (Hashimoto et al., 1991; Leonardi et al., 1995) in A549 cells and were able to produce plaques (Cromeans et al., 2008).

In this study, the adenovirus type 41 Tak strain - adapted to propagate and produce plaques in A549 - and adenovirus type 2 were used as models to assess the long term survival

of the Adenovirus group in artificially seeded environmental water samples under different temperatures, using a plaque assay as a quantitative method for virus counts and viability.

Temperature, dissolved oxygen, pH, total organic carbon, turbidity, chloride concentration, and the presence of indigenous microorganisms are all factors that have been shown to affect virus survival in surface and ground water (Yates et al., 1985; Gordon & Toze, 2003; John & Rose, 2005; Pedley et al., 2006). In general, in this study the ANOVA did not indicate a significant difference between the decay rates of each virus in the different surface and ground water samples for Ad2 and 41.

In this study most inactivation rates are reported as negative numbers. In some cases where the virus did not show any decay but an increase in concentration was measured, these numbers are positive. The smaller the absolute value of the decay rate/growth rate, the better the survival. It is important to note that viruses cannot replicate without a host, and thus, a positive survival rate as achieved in some measurements in this study, is an artifact of the detection method based on macroscopic plaques counts. Moreover, changes in cell passages, could affect the measurements in a long term study, such as the cell receptors expression and virus infectivity.

Temperature seems to play a key role in the survival of enteric virus in water samples as previously reported (Yates et al., 1985; Enriquez et al., 1995). Although viral survival studies are difficult to compare as they were carried out under different conditions. According to Table 2, the inactivation rates measured for Ad2 and 41 in this study were lower than previously reported inactivation rates. Enriquez et al. (1995) have shown that Ad40 and 41 in tap water had losses of 0.5 and 1.0 $\log_{10}$ after 55 days at 4°C and almost 2 $\log_{10}$ at room temperature, while in this study results of Ad2 at 19°C showed losses of 1.0 to 1.3 $\log_{10}$ in surface water and from 0.8 to 1.1 $\log_{10}$ in ground water and reduction of Ad41 with values ranging from approximately 0.9 to 1.2 $\log_{10}$ in surface water and from 0.9 to 1.1 $\log_{10}$ in ground water (Figure 1).

One possible explanation for this discrepancy in losses comparing these two studies may be the fact that different procedures were used to assess viral viability, in our case applying the plaque assay technique we were able to count plaques, quantifying virus in plaque forming units (PFU) in A549 cells, while the method applied in Enriquez et al. (1995) study was based on an estimative of virus infectivity ($TCID_{50}$) in PLC/PRF5. In addition, higher temperatures have been demonstrated to significantly decrease virus survival in groundwater (Pedley et al., 2006; Yates et al., 1985). In contrast with room temperature, the

highest temperature tested in this study was 19°C that was chosen based on the average temperature of drinking water at treatment plant units.

The resistance showed by both adenovirus tested in this study is in agreement with other studies that have shown a higher thermal stability of the enteric adenoviruses in comparison with polio 1 and hepatitis A virus in tap water (Enriquez et al., 1995). This increased resistance of adenovirus to environmental stresses may be associated with the double-stranded nature of their DNA, which if damaged, may be repaired by host cell DNA-repair mechanisms that in human cells can also repair a wide range of DNA damage (Bernstein & Bernstein, 1991). Therefore, when applying a cell culture technique in order to assess virus viability, the damaged viral DNA could be repaired by the host cell machinery. This could also be an explanation for the high resistance of these viruses, especially the enteric adenoviruses, for UV inactivation as previously reported in other studies (Thurston-Enriquez et al., 2003; Ko et al., 2005a; Yates et al., 2006).

In conclusion, using the same cell line and plaque assay for both Ad2 and Ad41 allowed direct comparison of results for the two viruses in only 7 days for each measurement. Although monitoring virus survival in relatively small volumes of water does not properly mimic all of the environmental factors in the environment, the data in this study provide valuable baseline information on adenovirus environmental stability and long-term survival. Moreover, this long-term survival should be considered in risk assessment models and drinking water management strategies.

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Table 1: Chemical analysis of water samples.

	Value					
Parameter	S1	S2	S3	GW1	GW2	GW3
Free chlorine (mg/l)	0	0	0	0	0	0
Total Chlorine (mg/l)	0	0	0	0	0	0
pH	8.2	8.06	8.3	7.85	7.9	7.65
Alkalinity (mg/l)	130	89	81	211	50	312
Total hardness (mg/l)	306	130	100	374	45	425
Ca hardness (mg/l)	70	76	68	91	36	320
Mg hardness (mg/l)	30	54	32	36	9	105
Nitrogen (nitrate) (mg/l)	0.3	0.75	0.72	10.4	1.5	15.3
Nitrogen (ammonia) (mg/l)	<0.03	<0.03	<0.03	<0.03	0.35	<0.03
Turbidity (NTU)	0.91	0.31	0.46	3.1	0.07	0.1

Table 2: Decay rates of Adenovirus type 2 and 41 in surface and ground waters incubated at 10 and 19°C.

	Inactivation Rate [Log10 (Nt/No)/day]				
Ad41	10°C	R-square 10°C	19°C	R-square 19°C	*P-value*
Ad41/SF1	-0.0015	0.4426	-0.0046	0.8423	0.0331[a*]
Ad41/SF2	0.0019	0.3504	-0.0026	0.2389	
Ad41/SF3	0.0014	0.2854	-0.0029	0.3422	
Ad41/GW1	0.0005	0.0291	-0.0033	0.3469	0.0003[b]
Ad41/GW2	0.0006	0.0357	-0.0024	0.2245	
Ad41/GW3	0.0008	0.0610	-0.0031	0.3527	
Ad2/SF1	-0.0061	0.5726	-0.0092	0.8441	0.0004[c]
Ad2/SF2	-0.0061	0.6426	-0.0102	0.8628	
Ad2/SF3	-0.0064	0.6741	-0.0102	0.8190	
Ad2/GW1	-0.0048	0.7193	-0.0068	0.8756	0.0064[d]
Ad2/GW2	-0.0040	0.6849	-0.0087	0.8966	
Ad2/GW3	-0.0050	0.7695	-0.0081	0.9318	

ANOVA analysis between 10 and 19°C of: [a] Ad41 in surface water (SF), [b] Ad41 in ground water (GW), [c] Ad2 in SF, [d] Ad2 in GW. * Indicates significance ($p<0.01$).

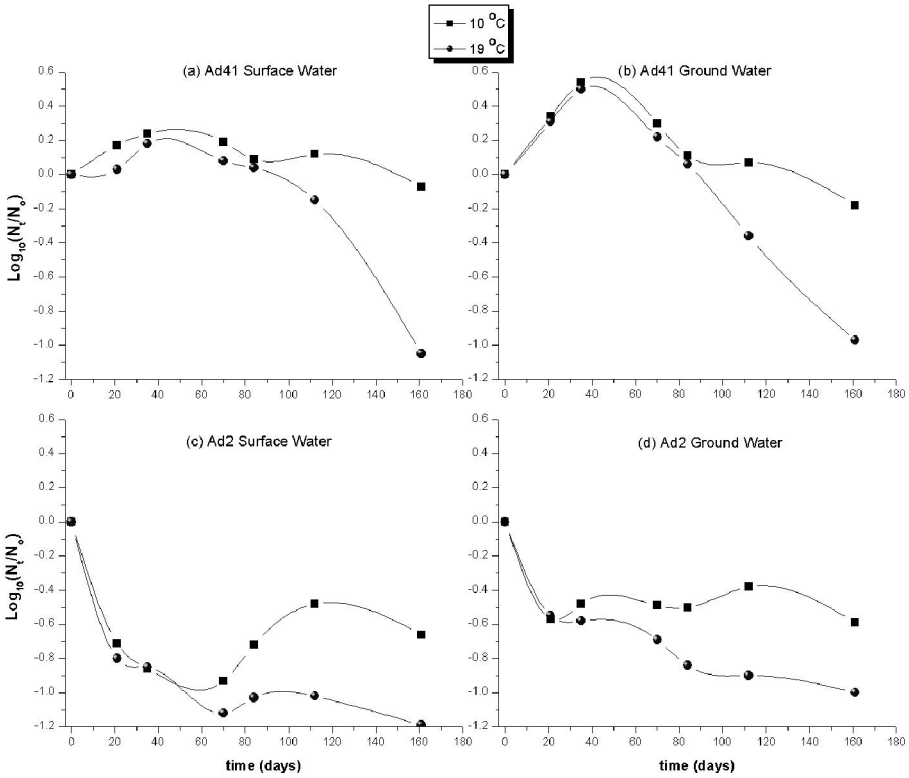

Figure 1: Survival of Adenovirus 2 and 41 in surface water and ground water.

5. DISCUSSÃO

5.1. Padronização de metodologias de concentração, detecção, quantificação e determinação da viabilidade de vírus entéricos (adenovírus, rotavírus, norovirus, e vírus da hepatite A) no ambiente aquático (águas e ostras).

A qualidade sanitária do ambiente aquático das áreas costeiras, de recreação, de cultivo de moluscos e de outras atividades relevantes à saúde humana inclui o monitoramento da contaminação fecal nesses locais. Qualquer tipo de poluição pode representar risco à saúde humana, porém a contaminação fecal representa um risco elevado, uma vez que patógenos potencialmente infecciosos aos seres humanos estão presentes nas fezes. Embora a detecção de indicadores bacterianos de contaminação fecal seja simples, sua ocorrência não correlaciona necessariamente à presença de patógenos virais, nem pode fornecer informações da potencial origem da contaminação ambiental (CALGUA et al., 2008).

Assim sendo, alguns aspectos favorecem a inclusão dos vírus como indicadores biológicos da qualidade das águas, tais como a resistência aos fatores ambientais e aos métodos de tratamento de água, sua circulação no ambiente sem um padrão característico de sazonalidade, sua presença em quantidades detectáveis e sua relação com surtos de gastroenterites no mundo (BOSCH, 1998; FONG; LIPP, 2005).

Tendo em vista a necessidade de se determinar os riscos de veiculação hídrica dos vírus tornou-se necessário o estabelecimento de métodos práticos e sensíveis para detecção de pequenas concentrações dos mesmos. No Brasil, existem alguns grupos de pesquisa e empresas que avaliam a presença de vírus entéricos humanos em águas de consumo e esgoto. Estes grupos estão localizados nos Estados do Rio de Janeiro (Instituto Oswaldo Cruz – FIOCRUZ), São Paulo (Companhia Estadual de Tratamento de Esgotos e de Abastecimento- CETESB e Universidade de São Paulo – USP), Espírito Santo (Universidade Federal do Espírito Santo- UFES), Goiás (Universidade Federal de Goiás – UFG) e Santa Catarina (Universidade Federal de Santa Catarina- UFSC). O Estado de Santa Catarina ainda não possui nenhum laboratório habilitado a realizar análises de detecção, quantificação e determinação da viabilidade viral em águas, sendo de fundamental importância que recursos humanos sejam formados nesta área.

Por essa razão, o presente estudo propôs padronizar e aplicar metodologias de concentração, detecção, quantificação e determinação viabilidade de vírus entéricos em águas ambientais e ostras de cultivo em Florianópolis, SC.

Sendo assim, no processo de otimização da metodologia de detecção de vírus em águas e ostras artificialmente inoculadas, foram utilizados como modelo os HAdV e o HAV, tanto na comparação de métodos de extração de ácidos nucléicos (capítulo 1), como na avaliação da recuperação viral a partir de diferentes matrizes, utilizando o método de filtração em membrana carregada negativamente (capítulo 2).

O estudo descrito no capítulo 1 teve como objetivo comparar três diferentes métodos de extração de ácidos nucléicos a partir de suspensões virais (como controle positivo) e de vírus inoculados em amostras de ostras e água destilada. Esses métodos de extração visam extrair e purificar ácidos nucléicos, removendo restos celulares e inibidores previamente à amplificação do material genético por PCR (GRIFFIN et al., 2001; LIPP et al., 2001). A eliminação de inibidores das reações enzimáticas e componentes tóxicos para células em cultura constitui um dos principais desafios a serem minimizados na área da Virologia Ambiental. Amostras concentradas de águas e moluscos, dependendo da matriz a ser analisada, além de concentrar os patógenos ali dispersos, entre eles os vírus, também concentram uma série de inibidores como proteínas, carboidratos, ácidos húmico e fúlvico, e outros compostos orgânicos (JOTHIKUMAR et al., 1998; SCHWAB et al., 1995; TAVARES et al., 2005; WYN-JONES; SELLWOOD, 2001).

Um dos métodos testados e amplamente utilizado na purificação de ácidos nucléicos foi desenvolvido por Boom et al. (1990) e baseia-se na utilização do isotiocianato de guanidina e colunas de sílica, apresentando-se como um método rápido e eficaz na remoção de inibidores e na recuperação de patógenos a partir de amostras ambientais (JIANG et al., 2001). Este método se mostrou eficiente em estudos prévios de recuperação de vírus, tais como adenovírus, vírus das hepatites A e E, e poliomavírus a partir de águas de consumo, de rio, de mar e de esgoto (BOFILL-MAS et al., 2006; PINA et al., 1998; PUIG et al., 1994).

Nas amostras analisadas no presente estudo, esse método também demonstrou uma boa eficiência na detecção de vírus em amostras de água destilada (HAdV=45 PFU), porém não foi eficaz na remoção dos inibidores frequentemente presentes nos tecidos de moluscos, não teria sido possível a detecção do HAdV. Nas mesmas amostras de ostras, o método de extração orgânico com fenol/Trizol® demostrou uma melhor recuperação (3.300 PFU para HAdV e 6.600 FFU para HAV), quando comparado aos outros métodos testados. Estes resultados sugerem que uma clarificação prévia com clorofórmio poderia ser realizada, o que de fato foi adaptado ao protocolo de purificação de ácidos nucléicos virais presentes em amostras de ostras nos posteriores estudos de amostras ambientais. Em amostas de água

destilada, o método de extração orgânica permitiu a detecção de 1,4 PFU do HAdV e 330 FFU do HAV.

No capítulo 1, também se utilizou o método de extração de ácidos nucléicos, antecedido por uma adsorção em cartões de papel filtro [Flinders Technology Associates (FTA®)]. Entretanto, somente com a utilização de suspensões virais puras, este método mostrou-se o mais eficiente tanto para HAdV (0,007 PFU) quanto para HAV (0,13 FFU). Esta técnica tem sido utilizada no processamento e detecção de DNA em medicina forênsica e em amostras de DNA obtidas em campo (DAN-MY et al., 2004; DESLOIRE et al., 2006; NANTAVISAI et al., 2007), porém não tem sido indicada para o processamento de amostras de água e alimentos para detecção de patógenos virais. Neste capítulo, sugeriu-se a utilização do FTA® para detecção de ácidos nucléicos partindo-se de amostras limpas, como cultura de células infectadas (suspensões virais), visto que amostras mais complexas necessitam de etapas extras de clarificação e purificação, como sugerido para o método da sílica testado.

Como conclusão, o estudo preliminar de avaliação de métodos de extração de ácidos nucléicos descrito no capítulo 1 apontou o método da sílica como o mais adequado para a detecção de vírus em amostras de água, o método de extração orgânica como o mais apropriado para extração de ácidos nucléicos a partir de amostras de moluscos e o método FTA® como o mais eficiente para extração a partir de amostras “limpas” como suspensões virais. Estes protocolos obviamente seriam os mais indicados quando métodos de extração comerciais, baseados tal como kits de purificação de ácidos nucléico**s,** não estiverem disponíveis nos laboratórios.

O estudo descrito no capítulo 2 teve como objetivo avaliar um método de adsorção-eluição utilizando membrana carregada negativamente para determinar a eficiência de recuperação de HAdV e HAV a partir de diferentes matrizes de água, combinando este método com o teste molecular qualitativo de PCR (*nested* RT-PCR e *nested* PCR). O método testado foi descrito por Katayama et al. (2002) e tem sido avaliado e aplicado a amostras de águas no Brasil e no mundo, da mesma forma que o PCR qualitativo tem sido descrito por diversos autores como uma ferramenta útil para detecção de vírus entéricos em amostras ambientais, tal como as de água de esgoto não tratado (FORMIGA-CRUZ et al. 2005), de rio (BORCHARDT et al. 2004), do mar (KATAYAMA et al. 2002) e de consumo (LEE et al. 2005).

Uma vez que poucas partículas virais estão presentes e dispersas no ambiente, se faz necessário a concentração de vírus a partir de grandes volumes de águas. Diferentes métodos têm sido descritos e utilizados, entretanto as recuperações virais nem sempre são similares,

uma vez que diversos fatores podem interferir na adsorção dos vírus nas membranas carregadas, como sais, cátions multivalentes e pH (ALBINANA-GIMENEZ et al., 2009; HARAMOTO et al. 2005, 2006, 2007; HSU et al. 2007; VICTORIA et al. 2009; VILLAR et al. 2006).

Membranas eletronegativas, incluindo os filtros Millipore HA® são comumente utilizados para concentração de vírus a partir de amostras de água utilizando o método de adsorção e eluição viral (APHA, 1998; USEPA, 2001). Neste método, o principal fator que pode interferir na eluição viral da membrana negativa é a interação elétrica e hidrofóbica dos vírus (HANZA et al., 2009). O tampão mais comumente utilizado para eluição viral a partir de membranas carregadas negativamente é uma solução alcalina de extrato de carne em tampão glicina, que é recomendado, por exemplo, pela Associação Americana de Saúde Pública (APHA) e pela Agência Americana de Proteção Ambiental (EPA) (APHA, 1998; USEPA, 2001), e que também tem sido utilizado no método de eluição viral a partir de membranas carregadas positivamente.

As interações hidrofóbicas com substâncias orgânicas presentes no extrato de carne apresentam um papel importante na eluição viral como descrito em trabalhos anteriores (LOGAN et al., 1980; YANO et al., 1993; SOBSEY et al., 1979). Entretando, os componentes orgânicos e inorgânicos presentes no eluato de extrato de carne são conhecidos por inibir tanto a etapa de síntese de cDNA a partir do RNA viral pela transcriptase reversa (e isso é crucial na detecção de vírus de genoma RNA) quanto a amplificação do DNA ou cDNA virais por PCR, que é a melhor ferramenta para detecção de vírus a partir de amostras ambientais (ABBASZADEGAN et al., 1993; KATAYAMA et al., 2002; HARAMOTO et al., 2004; SCHWAB et al., 1995). Deste modo, uma solução inorgânica pode ser considerado o melhor eluente em metodologias de concentração viral, onde as interações eletrostáticas devam ser consideradas como um dos principais mecanismos. Membranas carregadas negativamente podem ser consideradas mais apropriadas devido à força repulsiva entre a membrana negativa e os vírus carregados negativamente em condições alcalinas.

O método que utiliza membrana negativa testado no estudo do capítulo 2 tem como mecanismo o tratamento prévio da amostra de água com cátions bivalentes que interagem com os vírus facilitando a interação e a adsorção viral na membrana negativa. No passo seguinte, a lavagem da membrana com uma solução ácida (pH 3,0) transforma a carga viral para positiva. Uma vez que os vírus apresentam um alto ponto isoelétrico, espera-se que eles se liguem à membrana negativa e que os cátions sejam eluídos. Na subseqüente eluição

alcalina, a carga da superfície viral é convertida de positiva para negativa, permitindo a eluição do vírus da membrana carregada negativamente (KATAYAMA et al., 2002).

Cada método de concentração apresenta vantagens e desvantagens, sendo importante salientar que ainda não existe um único método capaz de recuperar todos os vírus entéricos com alta eficiência. Deste modo, é extremamente importante desenvolver, otimizar e avaliar métodos que possam detectar eficientemente vírus entéricos no ambiente aquático.

Os resultados apresentados no capítulo 2 mostraram que os métodos de concentração e detecção avaliados foram capazes de recuperar e detectar os genomas de HAdV e HAV em todas as amostras testadas, entretanto a recuperação viral na água do mar foi a que apresentou menor eficiência (10%). Este resultado está de acordo com as porcentagens de recuperação do mesmo método de concentração avaliado por outros autores para detecção do HAV, astrovírus e norovírus em água do mar (VILLAR et al. 2006, VICTORIA et al. 2009). Aparentemente, uma explicação para a baixa recuperação viral em água do mar usando métodos de adsorção-eluição, seria dificultada pela alta força iônica da ligação viral à membrana que é de natureza eletrostática (CALGUA et al., 2008; LUKASIK et al., 2000).

Outros métodos de concentração viral a partir da água do mar têm sido propostos recentemente com o objetivo de facilitar o processamento das amostras, isto é reduzir volume de amostras e custos, e obter uma melhor recuperação viral. Calgua e colaboradores (2008) desenvolveram um método que utiliza floculação com leite desnatado e obtiveram uma recuperação viral de HAdV em água do mar de até 49% utilizando PCR quantitativo. Esse protocolo de concentração de vírus em água do mar é baseado na ligação dos vírus às proteínas do leite desnatado pré-floculado, reduzindo o número de passos da concentração viral para apenas um, não requerendo a disponibilidade de equipamentos específicos e, desta forma, representando uma significante redução nos custos das análises.

Apesar da baixa recuperação em amostras de água do mar, os limites de deteção obtidos nestas amostras por *nested* PCR foram de 250 $TCID_{50}$/mL e 20 FFU/mL para HAdV and HAV, respectivamente. Em amostras de água destilada e de esgoto tratado a porcentagem de recuperação foi de até 100% quando comparado com controles positivos e os limites de detecção encontrados nestas amostras foram de 25 TCID50/mL para HAdV e 2 FFU/mL para HAV.

Desta forma, após os estudos realizados para a padronização do método de concentração viral utilizando amostras de águas de diversas matrizes semeadas com vírus, pôde-se prosseguir para o próximo objetivo do presente trabalho, que foi a aplicação destes métodos em amostras ambientais de campo.

5.2. Pesquisa de adenovírus humanos norovírus genogrupos GI e GII, rotavírus humanos do grupo A e vírus da hepatite A em águas ambientais e ostras de cultivo, durante o período de Junho de 2007 a Maio de 2008

Para a avaliação da aplicabilidade do método padronizado a partir de amostras ambientais semeadas com vírus, realizou-se a pesquisa de adenovírus humanos, norovírus dos genogrupos GI e GII, rotavírus humanos do grupo A e vírus da hepatite A em águas ambientais e ostras de cultivo de Florianópolis, SC, durante o período de Junho 2007 a Maio de 2008 (capítulos 3, 4 e 5).

O estudo descrito no capítulo 3 teve como objetivo detectar a presença, quantificar e determinar a viabilidade de vírus entéricos em amostras de água de esgoto tratado e lodo ativado, coletados durante um ano de uma estação de tratamento de esgoto localizada em Florianópolis, SC. O lodo ativado derivado da água de esgoto consiste em uma matéria orgânica rica e sua aplicação na agricultura como fertilizante tem sido discutida e aplicada em alguns países; entretanto, muitos organismos patogênicos de origem fecal podem estar presentes implicando num risco potencial à saúde humana (MONPOEHO et al. 2004).

Os resultados obtidos com a análise do lodo de esgoto fazem parte de uma dissertação de mestrado aprovada pelo PPG em Biotecnologia da UFSC e também se encontram no capítulo 3 deste trabalho (SCHLINDWEIN, 2008), cujos resultados foram submetidos para publicação em conjunto com os dados da presente tese, que avaliou amostras de água de esgoto.

A água de esgoto tratado, cujos resultados das análises fazem parte do presente trabalho, também tem sido alvo de muitas pesquisas que relatam a presença de vírus entéricos neste tipo de amostra. Estes vírus possuem certa resistência aos agentes físico-químicos de inativação, podem permanecer viáveis ou potencialmente infectantes durante meses na água, resistindo a condições ambientais adversas e aos processos de tratamento de água e esgoto, aplicados no controle bacteriano (TREE et al., 2003; SKRABER et al., 2004).

A presença de patógenos na água de esgoto tratado que é descartado no ambiente, como no Oceano Atlântico- que é o caso de Florianópolis, SC – pode acarretar em um desequilíbrio ambiental no ecossistema em contato, como também ser considerado um elevado risco à saúde humana. Os vírus entéricos são usualmente eliminados em grandes quantidades nas fezes, e os tratamentos de água de esgoto que não possuem uma etapa passo terciária (por exemplo, tratamento com UV) conseguem reduzir muito pouco esses níveis virais. Tratamentos com a produção de lodo de esgoto podem reduzir esses números em até

95%, porém os vírus presentes na água de esgoto ou no lodo, quando em contato com o ambiente, podem chegar a 100 PFU por litro (CARTER et al., 2005; GERBA et al. 1985; PINA et al., 2001), consistindo ainda um risco à população em contato.

Nos estudos descritos nos capítulos 4 e 5, amostras de ostras de dois locais de cultivo em Florianópolis (norte e sul da Ilha) e amostras de águas de consumo tratada e não tratada, de mar próximo às fazendas de cultivo de ostras, da Lagoa da Conceição, de uma boca-de-lobo na Lagoa da Conceição e de um córrego poluído numa comunidade de baixa renda, foram também avaliadas para a presença de vírus entéricos. Os resultados obtidos com a análise das águas e das ostras para detecção de NoV fazem parte da Tese de Doutoramento de Matias Victoria Montero do PPG em Biologia Parasitária da Fiocruz, RJ e se encontram no capítulo 4 deste trabalho. Os resultados das análises das águas e das ostras para detecção de HAdV, RV e HAV fazem parte do presente trabalho de tese e encontram-se no capítulo 5. As análises de NoV e dos demais vírus foram realizadas na mesma época e em conjunto e por isso ambos os artigos submetidos para publicação fazem parte dos resultados da presente tese descritos no capítulo 4.

Estudos anteriores relatam que mais de 140 tipos de vírus responsáveis por uma variedade de doenças podem estar presentes em águas ambientais, de superfície, de aquíferos e de consumo humano, afetadas pela contaminação fecal proveniente de esgotos (HURST et al., 1997; VILLENA et al., 2003). Entretando, apenas poucos vírus têm sido relacionados com surtos de gastroenterites relacionados com a água (ABBASZADEGAN et al., 1993; HOFFMAN et al., 2009; SCHWAB et al., 1995). Por outro lado, uma variedade de vírus entéricos tem sido associada com surtos de gastroenterites relacionados com alimentos. Vírus associados a alimentos são infeciosos a uma dose muito baixa e podem ser introduzidos em qualquer etapa da cadeia alimentar. Os moluscos, em particular, devido à sua capacidade de alimentação filtrante podem acumular patógenos presentes nas águas que os circundam e ao serem consumidos, na maioria das vezes, com nenhum ou pouco cozimento são identificados como uma das principais fontes de infecção alimentar de origem viral.

No presente estudo foram escolhidos para avaliação da contaminação viral em ostras e nas águas ambientais os adenovírus como modelo de vírus de genoma de DNA, e os rotavírus, vírus da hepatite A e os norovírus como modelo de vírus de genoma de RNA. A escolha destes vírus entéricos baseou-se tanto na sua incidência no ambiente aquático em estudos anteriores, como na importância epidemiológica no Brasil e no mundo.

Os iniciadores utilizados para amplificação de HAdV foram desenhados para detectar uma região conservada (gene *hexon*) de todos os tipos de adenovirus humanos (HERNROTH

et al., 2002). Durante o período estudado, o DNA de HAdV foi altamente prevalente e detectado através da *nested* PCR em todos os tipos de amostras ambientais, apresentando 100% de positividade em lodo de esgoto, 75% em esgoto tratado (conforme capítulo 3), 64,2% nas amostras de águas ambientais e em 87,5% das amostras de ostras (conforme capítulo 5). Estes resultados estão de acordo com aqueles obtidos em um outro que também investigou a presença de HAdV em ostras na mesma região geográfica (RIGOTTO et al., 2005) e com estudos que relatam uma alta prevalência destes vírus em águas ambientais sem um padrão de sazonalidade (FORMIGA-CRUZ et al., 2005; HUNDESA et al., 2006).

Os HAdV têm sido consistentemente detectados em esgoto não tratado e aparentemente têm apresentado a capacidade de sobreviver a tratamentos de esgoto, sendo frequentemente detectados em águas onde há pouca ou nenhuma contaminação por enterovírus. Este é um dos fatores que pode sugerir os HAdV como indicadores virais de contaminação fecal no ambiente aquático (ENRIQUEZ et al., 1995; PINA et al., 1998; WYN-JONES; SELLWOOD, 2001).

Os vírus de genoma de RNA analisados neste trabalho também foram encontrados nas amostras avaliadas, porém com um padrão bem distinto do encontrado para os HAdV. Os RV apresentaram por RT-PCR positividade de 33% em lodo, 41,6% em esgoto tratado (capítulo 3), 19% nas águas ambientais e 8,3% nas amostras de ostras (capítulo 5). Já os HAV apresentaram incidências de 25% tanto nas amostras de lodo como nas de esgoto tratado e de 8,3% nas amostras de águas ambientais. Os NoV foram detectados em 23% das águas ambientais (incluindo as amostras de esgoto tratado). Nas amostras de ostras, não foi detectada a presença de HAV nem de NoV. Estes resultados podem ser devido à baixa endemicidade destes vírus na região Sul do Brasil.

Esta maior positividade de HAdV perante os outros vírus em todas as amostras já era esperada devido à sua maior resistência aos fatores ambientais e sua ampla incidência no ambiente, já relatadas em estudo anterior (HUNDESA et al., 2006). Todos os vírus demostraram uma elevada presença nas amostras de lodo, de esgoto tratado e do córrego poluído, sendo também menos frequentes em amostras de água de consumo humano. É importante ressaltar que todas as amostras positivas para NoV, RV-A e HAV também foram positivas para HAdV, o que sugere que a detecção de vírus de RNA não foi inibida por níveis naturais de HAdV nas amostras estudadas.

Hoje em dia está bem estabelecido que a detecção de vírus por técnicas moleculares indica apenas a presença do genoma viral e não fornece informações de infectividade, o que está diretamente relacionado com risco à saúde humana (CROMEANS et al., 2008; Ko et al.,

2005b; REYNOLDS et al., 2004). O PCR integrado à cultura cellular (ICC-PCR) tem se mostrado um método rápido e sensível para detecção de alguns vírus entéricos em amostras ambientais nas quais a concentração viral é relativamente baixa e os níveis de inibidores altos (REYNOLDS et al., 2004).

Neste estudo o ensaio de ICC-PCR foi aplicado na tentativa de se verificar o potencial infeccioso dos vírus entéricos encontrados nas amostras ambientais, com exceção dos norovírus que não são adaptáveis à cultura celular. Dentre as amostras positivas para HAdV, a grande maioria também mostrou-se viável através do ensaio de ICC-PCR, com valores de 100% para lodo de esgoto, 66,6% em esgoto tratado e 83,8% nas amostras de águas ambientais. Os RV foram confirmados viáveis em 25% das amostras de lodo e 12,5% das amostras de águas ambientais, e os HAV em 16,7% das amostras de lodo e 66,6% das de esgoto tratado. Através deste ensaio, nenhuma amostra de esgoto apresentou positividade para RV, assim como nenhuma foi positiva para HAV nas amostras ambientais. Esta baixa viabilidade de RV em esgoto, assim como a ausência de HAV viáveis nas amostras ambientais pode não estar relacionado com a infecciosidade destes vírus, mas muito provavelmente devido ao fato de que os genomas virais encontrados são provenientes de vírus fastidiosos, não adaptados à infecção *in vitro* das células.

No método de PCR associado à cultura celular (ICC-PCR) aplicado no presente estudo, a detecção dos vírus viáveis foi realizada a partir da extração dos ácidos nucléicos presentes no sobrenadante (meio de cultura) celular, partindo-se do princípio de que apenas os vírus infeciosos inoculados nas células permissivas se replicarão e poderão estar presentes no sobrenadante, uma vez que após a inoculação da amostra por 1h, esta é removida e as células lavadas com tampão (PBS) com o objetivo de remover partículas virais não adsorvidas. As técnicas de cultura celular associadas a PCR também tem suas limitações, como por exemplo, a possível amplificação de partículas virais incompletas, de DNA ou RNA livre de capsídeo viral, portanto não infecciosos.

Recentemente, um novo método molecular foi desenvolvido para detecção de HAdV infecciosos combinando a cultura celular com mRNA RT-PCR, seguido de *nested* PCR (KO et., 2003) ou PCR quantitativo (KO et al., 2005b). Nestes métodos adenovírus infecciosos puderam ser detectados a partir da sua habilidade em produzir mRNA durante a replicação em cultura celular e, desta forma, pequenas quantidades de vírus víaveis podem ser detectadas e quantificadas através dessa técnica que tem se mostrado promissora.

Além da presença e da viabilidade viral, a quantificação de HAdV, HAV e NoV nas águas ambientais e nas ostras previamente positivas por *nested* PCR ou RT-PCR, também foi

avaliada no presente trabalho. Por PCR quantitativo, os HAdV foram avaliados em todos os tipos de amostras (capítulos 3 e 5), as médias de cópias do genoma encontradas foram de 9,8 x 10^4 gc/L em esgoto tratado, 1,87 x 10^7 gc/L em lodo, 9,8 x 10^6 gc/L em águas ambientais e 1,2 x 10^5 gc/g em amostras de ostras. Os NoV foram avaliados em todas as amostras positivas (capítulo 4), exceto no lodo de esgoto, sendo que os valores encontrados foram menores do que os de HAdV: 1,8 x 10^2 gc/L (sorotipo G1) e 1,0 x 10^3 gc/L (sorotipo G2) em esgoto tratado e 1,1 x 10^2 gc/L (GI) e 8,1 x 10^3 gc/L (GII) em águas ambientais. Os HAV foram avaliados apenas nas amostras de lodo e de esgoto no estudo apresentado no capítulo 3. As médias encontradas para os dois locais foram a mesma: 1,2 x 10^4 gc/L.

A partir dos resultados encontrados nos estudos de quantificação dos genomas virais nas amostras no ambiente aquático, concluímos que os HAdV foram os mais prevalentes, seguidos de HAV e NoV. Estes níveis de vírus encontrados podem representar um elevado risco à população em contato com o ambiente analisado.

5.3. Estudo do decaimento da infectividade de adenovírus humanos em amostras de águas, mantidas em diferentes temperaturas, por períodos de tempo variáveis.

A longa persistência e resistência dos adenovírus no ambiente têm aumentado o interesse no monitoramento da presença destes vírus no ambiente aquático. A avaliação do risco à saúde humana de certos patógenos presentes em águas de consumo requer o conhecimento do tipo e concentração do patógeno, da dose infecciosa e das características de sobrevivência em águas naturais. Poucos estudos relacionados às características de sobrevivência de adenovírus em águas naturais foram conduzidos, principalmente devido à dificuldade do cultivo *in vitro* de algumas espécies destes vírus, especialmente os entéricos (MENA; GERBA, 2009). Entretanto, Cromeans et al. (2008) recentemente desenvolveu um ensaio de placa de lise em células A549 utilizando cepas de adenovírus entéricos (HAdV 40/41) capazes de infectar estas células *in vitro* e produzir placas de lise, permitindo sua quantificação.

O estudo descrito no capítulo 6 do presente trabalho teve como objetivo realizar um estudo de decaimento da infectividade de HAdV tipos 2 e 41 em águas de superfície e subterrânea, mantidas em diferentes temperaturas por períodos de tempo variáveis, utilizando o ensaio de placa de lise para quantificar os vírus sobreviventes.

Alguns fatores têm sido relacionados com a sobrevivência dos vírus em águas de superfície e subterrâneas como: temperatura, oxigênio dissolvido, pH, turbidez, concentração de cloro e presença de microorganismos (GORDON; TOZE, 2003; JOHN; ROSE, 2005; PEDLEY et al., 2006; YATES; GERBA; KELLEY, 1985). Entretanto, neste estudo a análise por ANOVA não demonstrou diferença significativa entre as taxas de decaimento de ambas as cepas de HAdV nas diferentes amostras águas analisadas.

A sobrevivência viral avaliada foi baseada no cálculo de regressão linear, sendo que quanto menor o valor absoluto da taxa de decaimento/crescimento, melhor a sobrevivência viral. Neste estudo a maioria das taxas de inativação viral alcançadas foram descritas como números negativos (significam decaimento), porém em alguns casos onde os vírus não apresentaram nenhum decaimento, mesmo após um longo período de incubação, as taxas obtidas foram descritas como números positivos (significam aumento). Este fato pode ser considerado um artefato do método de detecção, que é baseado na contagem macroscópica das placas de lise. Além disso, mudanças na passagem celular e a expressão de receptores, poderiam afetar as leituras em um estudo a longo prazo.

As taxas de inativação de HAdV2 e 41 obtidas neste trabalho foram menores do que as obtidas em trabalhos anteriores. Enriquez et al. (1995) demonstrou o decaimento de adenovírus entéricos de até 1 $\log_{10}$ após 55 dias à 4°C e de quase 2 $\log_{10}$ à temperatura ambiente pelo mesmo período. Neste estudo, após 161 dias, os decaimentos de HAdV2 encontrados foram de até 1,3 $\log_{10}$ em águas de superfície e de até 1,1 $\log_{10}$ em águas subterrâneas, ambas mantidas à 19°C. A redução de HAdV41 foi de até 1,2 $\log_{10}$ em águas de superfície e até 1,1 $\log_{10}$ em águas subterrâneas na mesma temperatura.

Uma possível explicação para essa discrepância de valores de decaimento comparando esses dois estudos pode ser devido à diferença entre os métodos aplicados para avaliar a viabilidade viral, que neste caso foi através de placa de lise em células A549, na qual a quantificação viral é expressa em unidades formadoras de placa (PFU). O método aplicado no estudo de Enriquez et al. (1995) foi baseado na estimativa de vírus infecciosos em células PLC/PRF5 expressa em $TCID_{50}$. Além disso, os dois trabalhos avaliaram a sobrevivência dos adenovírus em temperatura distintas: 4°C e temperatura ambiente (ENRIQUEZ et al., 1995) e 10°C e 19°C (capítulo 6).

A sobreviência dos adenovírus em águas naturais observada no presente estudo, está de acordo com outros estudos que mostram uma maior estabilidade destes vírus no ambiente em comparação a outros vírus entéricos (MENA; GERBA, 2009). Tanto a sobrevivência quanto a estabilidade dos adenovírus no ambiente podem estar associadas à natureza do

genoma destes vírus (DNA dupla fita), que em caso de dano, poderia ser reparado pela maquinaria de reparo de DNA da célula hospedeira (BERNSTEIN; BERNSTEIN, 1991). Deste modo, ao aplicar métodos de cultura de células para avaliar a viabilidade viral, o DNA danificado poderia ser reparado pela célula hospedeira. Esta poderia ser também uma possível explicação para a alta resistência dos adenovírus, especialmente os entéricos aos processos de inativação viral por UV previamente descritos em estudos anteriores (KO et al., 2005a; THURSTON-ENRIQUEZ et al, 2003; YATES et al., 2006).

No presente capítulo, a utilização de uma mesma linhagem celular e um mesmo método de quantificação viral (ensaio de placa de lise) para avaliação de HAdV2 e HAdV41, permitiu a comparação direta dos resultados obtidos. Embora o monitoramento da sobrevivência de vírus em volumes pequenos de água possam não mimetizar propriamente as condições ambientais, os dados apresentados neste estudo fornecem informações valiosas referentes à estabilidade termal dos adenovírus em longo prazo que poderão ser consideradas em modelos de avaliação de risco e estratégias de gestão da água potável.

6. CONCLUSÕES

I. O estudo preliminar de avaliação de métodos de extração de ácidos nucléicos descrito no capítulo 1 apontou o método da sílica como o mais adequado para detecção de vírus em amostras de água, o método de extração orgânica como o mais apropriado para extração de ácidos nucléicos a partir de amostras de moluscos e o método FTA como o mais eficiente para extração a partir de amostras "limpas", tais como suspensões virais.

II. Os resultados apresentados no capítulo 2 mostraram que os métodos de concentração (concentração/eluição em membrana negativa) e detecção (PCR qualitativo) avaliados foram capazes de recuperar e detectar o genoma de HAdV e HAV em todas as amostras testadas. Entretanto, a recuperação viral na água do mar foi a que apresentou menor eficiência.

III. As metodologias testadas e estabelecidas foram capazes de determinar a presença, a quantificação e a viabilidade de vírus entéricos em amostras de águas (capítulos 3, 4 e 5).

IV. Todas as amostras testadas e positivas para RV-A, HAV e NoV também foram positivas para HAdV, que foi o vírus que apresentou maior prevalência no ambiente aquático, tanto em águas ambientais como nos moluscos analisados.

V. Demonstrou-se elevada presença dos vírus entéricos avaliados (HAdV, RV-A, HAV, NoV) nas amostras de lodo, de esgoto tratado e do córrego poluído, sendo menos freqüentes em amostras de água de consumo humano.

VI. O estudo da sobrevivência de HAdV em águas de superfície e subterrâneas demostrou alta estabilidade destes vírus a longo prazo nas temperaturas testadas.

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Chapter 4

DETECTION OF ADENOVIRUSES AND HEPATITIS A VIRUS IN WATER SAMPLES AND OYSTERS: USE OF THREE DIFFERENT NUCLEIC ACID EXTRACTION METHODS

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ABSTRACT

Enteric viruses may be naturally present in aquatic environments and human and animal wastes excrete over 100 types of pathogenic viruses. These viruses can reach the environment through different sources including groundwater, seawater, aerosols emitted from sewage treatment plants and drinking water. They are important because of their public health implications as well as frequent occurrence in many aquatic environments. It is well known that mollusks grown in seawater contaminated by urban wastewater present health risk if consumed raw or lightly cooked. Seafood is implicated in 10-19% of 76 million cases of food born diseases reported every year. Human adenovirus has been proposed as indicators of human viral contamination of environmental and wastewater samples because they are often associated with other human enteroviruses such as Hepatitis A virus. Microbiological quality of water and seafood is often based on quantitative methods for enumeration of fecal bacterial indicators including coliform and *Escherichia coli*. However, several reports have shown that such read out cannot predict the presence of enteric viruses in water. The State of Santa Catarina is a major producer of mollusks in Brazil due to excellent geographical conditions for marine organism cultures, especially bivalve mollusks such as the oyster species *Crassostrea gigas*. In recent years, however, increased pollution of mollusk culture habitats by sewage discharged from coastal communities has led to contamination of cultured mollusks by bacterial and viral pathogens. The increasing pollution of the coastal waters of Santa Catarina State requires monitoring of cultured bivalve mollusks to ensure health safety standards. To

expand commercial bivalve mollusk culture in Brazil, a safe product with high commercial value must be guaranteed by specially providing a good source of water for mollusk culture. Concentrating viruses in water by adsorption and subsequent elution from electronegative membranes is a worldwide method. In the present study, we aim to optimize the methodology for adenovirus and hepatitis A virus detection in artificially seeded water by using electronegative membranes and in artificially seeded oysters.

INTRODUCTION

Enteric viruses have been detected in environmental samples worldwide and are responsible for many human diseases as gastroenteritis, meningitis, miocarditis and hepatitis. Gastroenteritis is responsible for millions of deaths per year, mostly in children (Tortora et al., 2000), and is related to many bacterial (*Escherichia coli*, *Shigella* sp., *Salmonella* sp), protozooan (*Cryptosporidium* spp, Giardia sp) and viral pathogens (enteric viruses) (Koopmans et al., 2002; Souza et al., 2003; Greinert et al., 2004).

Adenovirus (Advs) is a member of the Adenoviridae family and is grouped in 2 genera: *Mastadenovirus* and *Aviadenovirus*. The first genus is isolated from mammals and the second, from avian (Flint et al., 2000). Advs are nonenveloped, range from 90-100nm in diameter, and consist of linear double stranded DNA (Kapikian and Wyatt, 1992). The Adenoviridae has 51 human serotypes, classified in 6 subgenera (A-F) (Allard et al., 2001; Avellon et al., 2001). Serotypes 1, 2, 5 and 6 are classified in subgenus C and are considered endemic, infecting adenoid and tonsils and can be isolated from fecal samples (Allard et al., 2001). The last group, subgenus F is the second most important viral pathogen of childhood gastroenteritis after rotavirus (Pintó et al., 1995, Medeiros et al., 2001; Soares et al., 2002). Clinical symptoms associated to Advs are diarrhea, vomiting and fever. Comparing to rotavirus infections, the Advs diarrhea is less severe, and the viruses are shed for extended periods in feces, urine and respiratory secretions of infected persons (Crabtree et al., 1997). The adenovirus type 5, the nonenteric adenovirus strain that accounts for 11% of clinical adenovirus cases reported to WHO, is also frequently detected in aquatic environments (Suttle and Chen,1992). Serological surveys show that 50% of older children as well as adults are immune for Advs infections (Lees, 2000). Advs are resistant to chemical and physical disinfectants such as chlorine and UV radiation, as well as pH variations. These characteristics allow them to survive in environment for long periods (Puig et al., 1994; Pina et al., 1998; Jiang et al., 2001).

Hepatitis A Virus (HAV) is a nonenveloped virus member of the family Picornaviridae, with a linear single-stranded positive sense RNA genome and 27 nm diameter. Due to its conserved genome, the strains isolated worldwide correspond to only one serotype, which is organized into six genotypes. There are four human (I, II, III and IV) and three simian (IV, V, VI) genotypes (Costa-Mattioloi et al., 2003). HAV is the first etiologic agent of acute hepatitis in the world and can cause substantial morbidity, principally in developing countries (De Paula et al., 2007). HAV infection occurs by fecal-oral route through with ingestion of contaminated water or food (Cuthbert, 2001). The viral particle is extremely resistant to degradation, can survive up to one month at room temperature and is only partially inactivated at 60°C for 12hs (Mbith et al., 1991). Due to the occurrence of a number of

outbreaks as well as isolated cases, HAV is currently recognized as a significant waterborne human pathogen (De Paula et al., 2007).

Diseases caused by the consumption of bivalve mollusk shellfish containing pathogenic viruses of human origin are frequently reported, particularly related to raw oysters (Myrmel et al., 2004). The shellfish are able to concentrate viral particles as a consequence of their filter-feeding process and therefore, when water is contaminated with human feces, viral pathogens may get trapped into the shellfish (Croci et al., 2007). The predominance of oysters, clams and mussels as vehicles of seafood-borne disease is probably due two factors: 1) the filter-feeder property of the shellfish which accumulate selectively the marine microflora, in addition to bacteria and viruses and 2) shellfishes are normally eaten raw or followed by a very mild heat cooking process (Feldhusen et al., 2000; Muniain-Mujika et al., 2002; Murchie et al., 2005).

Traditionally, coliform bacteria and *Escherichia coli* have been used as indicators of the sanitary quality of shellfish, and this has led to success in the prevention of shellfish-borne infections by fecal bacteria. However, it has been clearly established that bacterial standards do not always reveal the presence of viruses in shellfish (Formiga-Cruz et al., 2002). Human health problems associated with shellfish consumption are well described and contaminating viruses have been linked to nearly all episodes of gastroenteritis as well as outbreaks of illness related to consumption of contaminated shellfish (Lees, 2000). Many viruses transmitted by the fecal-oral route are widely prevalent in the community, and infected individuals can shed millions of viral particles in their feces. Consequently, viruses of many types occur in large numbers in sewage (Kingsley and Richards, 2001).

In viral contaminated environmental samples such as shellfish and waters, low levels of infectivity are expected. Thus, there is a clear need for a practical test for viral contamination of shellfish. Unfortunately, wild-type virus strains including HAV and noroviruses (NV) are fastidious and very difficult to culture *in vitro* (often without apparent cytopathic effects). Accordingly, PCR-based detection of viral nucleic acid is a highly sensitive technique and is capable of detecting viruses that are present in low numbers in environmental samples and are difficult to be maintained *in vitro* cell culture assays (Kinsgley and Richard, 2001). Nevertheless, successful virus genome amplification from these samples may also be a problem. Humic acids, heavy metals and phenolic compounds may inhibit activity of the polymerase enzyme (Croci et al., 2007). An alternative approach to detect viruses in shellfish without the interference of inhibitors has been the use of dissected digestive tract followed by direct purification of intact virions from tissue prior to amplification-based methods (Jaykus et al., 1996; Croci et al., 2007).

In highly populated coastal areas, large amounts of treated and sometimes untreated human wastewaters are discharged into marine environments resulting in increases in pathogen concentrations, which in turn serve as sources for transmission of many diseases (Lemarchand et al., 2004). Santa Catarina State is a major producer of mollusks in Brazil due to excellent geographical conditions for marine organism cultures, especially bivalve mollusks, such as the oyster species *Crassostrea gigas* (Oliveira-Neto, 2005). In recent years, however, increased contamination of mollusk culture habitats by sewage discharged from coastal communities has led to contamination of cultured mollusks by bacterial and viral pathogens (Coelho et al., 2003; Rigotto et al., 2005; Sincero et al., 2006). Detection of viruses in water where mollusks are cultured is an important step to ensure high quality of this product.

The basic steps for virological analysis of environmental waters are: sampling, viral concentration (and purification) and detection by molecular and cell culture-based methods. Cell culture had been a common method to isolate viruses in water until the early 1990s (Johl et al., 1991; Havelaar et al., 1993). The traditional cell culture assay method of pathogenic viral detection is impractical for routine monitoring, since it requires large sample volumes and days to weeks to obtain the results. Also, some viruses are not adapted to cell culture (Lipp et al., 2001; Theng-Theng Fong and Erin K., 2005). Some recent studies have used integrated cell culture and PCR, consisting of a cell culture step followed by PCR, in order to detect infectious viruses in environmental samples when virus concentrations are low and the levels of inhibitory compounds are high. (Chapron et al., 2000; Greening et al., 2002; Ko et al., 2003; Rigotto et al., 2005) While such approach presents some advantages and may be suitable for research studies, it is also more costly and time-consuming than direct reverse transcriptase PCR (RT-PCR) or PCR. Enzyme immunoassay has been used, but to a lesser extent, to determine the presence of the rotavirus antigen in water samples and sewage samples (Dahling et al., 1993; Kittigul et al., 2000). Molecular techniques have been used extensively to detect enteric viruses from environmental samples since the early 1990s. More recently, PCR has become a major tool for detection, and various types of viruses have been isolated in surface water by PCR, including fastidious viruses, such as NVs and HAV (Gilgen et al., 1997; Haramoto et al., 2004). PCR can also contribute to epidemiological studies because it is capable of differentiating specific viruses and also different genotypes of the same virus through the use of specific primers (Pina et al., 1998; Jiang et al., 2001; Katayama et al., 2002; Theng-Theng Fong and Erin K., 2005).

Since only few virus particles are present in water samples, virus detection always requires the prior concentration of the water sample (Gilgen et al., 1997; Abbaszadegan et al., 1999; Lipp et al., 2001). The concentration step involves filtration of water samples with adsorption of the viruses to charged microporous membranes, followed by virus elution. An additional ultrafiltration step may be required depending on the volume of the eluant (Nupen and Bateman, 1985; APHA, 1995).

Different types of filtration methods and filters, such as vortex flow filtration, tangential flow filtration, acid flocculation cartridge filters (electropositive or electronegative), glass fiber filters and glass wool filters, have been used to collect and concentrate viral particles from water samples (Jiang et al., 2001; Lipp, et al., 2001; Griffin et al., 2003). Virus recoveries using water samples from different sources are not always standardized due to the fact that viral adsorption to the charged membranes may be influenced by salts, multivalent cations, or acid conditions (Sobsey et al., 1973; Lukasik et al., 2000). Because of the tiny diameter of viral particles, mechanical filtration is often not possible; therefore, manipulation of charges on the virus surface, using pH changes to maximize their adsorption to charged filters is required. (Theng-Theng Fong and Erin K., 2005; Katayama et al., 2002).

Adsorption-elution of viruses with electropositive membranes (i.e., 1MDS Zetapor Virosorb [CUNO, Meriden, CN]) is considered one of the most useful methods to concentrate virus from water samples (APHA, 1995). These filters require no manipulation of pH because most enteric viruses are negatively charged in nature. However, virus recovery from seawater is not always efficient because of low viral adsorption to the positively charged membrane due to the influence of salts (Katayama et al., 2002; Lukasik et al., 2000). In these conventional concentration methods, a beef extract solution (1–3%, pH 9–11) has been used to elute viruses adsorbed to the filter. However, organic and inorganic compounds in beef

extract solution are known to inhibit cDNA synthesis and PCR amplification (Abbaszadegan et al., 1993).

Electronegative filters show higher virus recoveries from marine water and waters of high turbidity than do electropositive filters (Enriquez et al., 1995; Lipp, et al., 2001; Lukasik et al., 2000; Katayama et al., 2002). Viruses in water adsorb to an electronegative filter in the presence of multivalent cations (Lukasik et al., 2000). In previous studies developed by Katayama et al. (2002), Haramoto et al. (2004) and Haramoto et al. (2007), two methods for virus concentration were performed based on the electrostatic interaction among viruses and multivalent cations (Mg^{2+} or Al^{3+}) using an electronegative filter.

Ultrafiltration methods such as vortex flow filtration (VFF) and tangential flow filtration (TFF) are alternatives to adsorption-elution techniques (Theng-Theng Fong and Erin K., 2005). However, both VFF and TFF are less cost-and time-effective than adsorption-elution because of the high cost of equipment (Haramoto et al., 2004). After concentration and elution, water samples need to be reconcentrated in order to reduce the final volume of samples (Jiang et al., 2001; Lipp, et al., 2001; Theng-Theng Fong and Erin K., 2005). Commonly used secondary concentration methods include organic flocculation, PEG precipitation and centrifugal ultrafiltration (ultraconcentration based on a molecular weight cutoff, such as with Centriprep YM-30 or YM-50 and Amicon Ultra-15 concentrator columns [Millipore, Billerica, MA]) (EPA, 1996; Katayama et al., 2002; Haramoto et al., 2004). Amicon is a polyacrylonitrile-based ultrafiltration membrane unit used for concentration of biological samples containing antigens, antibodies, enzymes and microorganisms (Milipore, 2007). This membrane showed to be efficient for HAV recovery from artificially seeded distilled water (Vaidya et al., 2004).

Nucleic acid can be extracted by many different protocols such as organic extraction by phenol, silica method, FTA® cards and commercial kits. Trizol LS Reagent® is a ready-to-use reagent for the isolation of total RNA from cells and tissues. It is a mono-phasic solution of phenol and guanidine isothiocyanate (GuSCN). GuSCN is a powerful agent in the purification and detection of DNA and RNA because of its potencial to lyse cells and inactivate nucleases (Boom et al., 1990). FTA® card is a chemically treated filter paper developed for collection and room temperature storage of biological samples for molecular analysis (Purvis et al., 2006). The classical protocol for nucleic acid elution from the card is using FTA® Purification reagent, but an alternative elution solution proved to be also efficient (Bhattacharya et al., 2004). The silica method is a rapid and simple method for purification of nucleic acids and was described by Boom et al. (1990). In the presence of high levels of GuSCN nucleic acids particles bind to silica. After a wash step with a buffer containing GuSCN the samples are washed by ethanol and acetone. It is less prone to DNA and RNA sample-to-sample contamination because the nucleic acids bind to silica. In the present study, the cation-coated filter concentration method described by Haramoto et al. (2004) using electronegative filters, was adapted to evaluate recovery yields of Advs and HAV in distillated water samples. Reconcentration was made with the Amicon Ultra 15 system. The three methods here described for nucleic acids purification were used to evaluate the detection of Advs and HAV artificially seeded in oysters and distillated water. PCR and RT-PCR were used for molecular viral detection.

MATERIAL AND METHOD

Viruses and Cells:

HAV (strain HM 175) and Human adenovirus Ad5 (genogroup C, serotype 5) were propagated in a continuous line of fetal FRHk-4 cells (Sincero et al., 2006) and Hep-2 cells (Rigotto et al., 2005), respectively. Cells were cultured in Eagle's minimal medium (MEM-Sigma), supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL, Life Technologies do Brazil Ltda, São Paulo, SP, Brazil), streptomycin (100µg/ml), penicillin G (100U/ml), and amphotericin (0,025µg/ml) (Gibco-BRL).

For determination of virus titers, indirect immunofluorescense assay was used for HAV viruses, as previously described (Barardi et al. 1998) and lysis plaque assay was the method of choice for Ad5 titer determination (Burlenson et al., 1992). The titer of the HAV was 4.0 x 10^6 FFU/ml and Ad5 was 7.0 x 10^6 PFU/ml.

Oysters:

Oysters *Crassostrea gigas* were obtained from a farm in Florianopolis/Brazil. Gastrointestinal tracts of 12 oysters were gathered and homogenized with a shaft blender (Ultra-Turrax T-25 IKA®). A final volume of 2.0ml of the homogenate was seeded, separately, with 500µl of virus stocks. The homogenate was then added to 4.0 ml of 10% tryptose phosphate broth (TPB - 20.0g triptose, 2.0g glucose, 5.0g NaCl, 2.5g Na_2HPO_4 pH7.3) prepared in 0.05 M glycine pH 9.0 and shaked for 30 min at room temperature and the same volume of chloroform/butanol (1:1) was added. Following centrifugation at 10,000 xg for 20 min at 4°C, the supernatant was added to a 24% polyethylene glycol solution (PEG 6000) in NaCl 1.2M to a final concentration of 12%. The mixture was stirred for 2h at 4°C and centrifuged at 10,000 xg for 20 min at 4°C. The pellet was suspended in 3.0 ml of sterile MilliQ water, and 500µL were reserved for nucleic acids extraction.

Water Concentration:

Concentration of viruses in water was performed by adsorption in an electronegative membrane and subsequent elution as described by Katayama et al. (2002). For method standardization, two liters of distilled water were seeded with 1.0ml of Ad5 or 1.0 ml of HAV strain HM 175. Water samples were added with 10.0ml of $AlCl_3$ (0.1M) and pH adjusted to 5.0 using HCl. Water samples were filtered using a vacuum pump fitted with a type AP20 membrane and subsequently filtered using a type HA 0.45-µm negatively charged membrane filter (Millipore Corporation, Bedford, MA, USA). The membrane was then rinsed with 350.0 ml of H_2SO_4 (0.5mM, pH 3.0) to remove cations, and adsorbed viruses were eluted from the membrane with 10.0 ml of NaOH (1.0mM, pH 10.5). The membrane was recovered in a tube containing 50µl of H_2SO_4 (50mM pH 1.0) plus 50µl of 100X Tris EDTA buffer, pH 8.0 for neutralization. The filtrate was then concentrated by Amicon Ultra 15 system (Millipore) at

3.500xg, for 2 min resulting in a 2.0ml final volume and 500µL were utilized for nucleic acids extraction.

Nucleic Acids Extraction:

Organic-Based Extraction Method

For DNA/RNA extraction, 500µl of both, water and oyster processed samples, were treated with proteinase K at final concentration of 200µg/ml, in a digestion buffer composed by Tris-HCl, pH 7.5, sodium dodecyl sulfate (SDS) and ethylenediaminetetraacetic acid (EDTA) at final concentrations of 10mM, 0.5% and 5mM, respectively, for 30min at 56°C. The oyster samples were then treated with cetyltrimethylamonium bromide (CTAB) and NaCl at final concentrations of 1.3% and 0.4M, respectively, and incubated at 56° for 30min. After that, DNA was extracted from all Advs seeded samples twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), followed by one extraction with an equal volume of chloroform. The nucleic acids were precipitated from the aqueous phase using one volume of 100% isopropanol for 10 min at room temperature. The resulting pellets were washed with chilled 70% ethanol, dried at room temperature and suspended in 20µl of sterile MilliQ water.

RNA was purified from HAV seeded samples with Trizol® LS reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacture's instructions. Briefly an equal volume of Trizol® LS reagent was added in each sample, homogenized and incubated at room temperature for 5min. After incubation, 400µl of chloroform were added, homogenized, and incubated for 10min at room temperature and centrifuged for 15min at 12,000×g, 4°C. The aqueous phase was transferred to a fresh microtube and RNA was precipitated with one volume of 100% isopropanol for 10 min. The resulting pellets were washed with chilled 70% ethanol, dried at room temperature and suspended in 20µl of sterile diethylpyrocarbonate (DEPC)-treated water. Purified nucleic acids were stored at -80°C for RT-PCR assays.

Extraction with Silica Method

Extraction with silica was performed as described by Boom et al. (1990), with the following modifications. Four hundred microliters of both water and oyster processed samples were added to 800µl of lysis buffer L6 (5.25 M guanidinium thiocyanate (GuSCN), 50 mM Tris- HCl, pH 6.4, 20 mM EDTA, 1.3% [wt/vol] Triton X-100) and incubated at RT for 10min. Subsequently, fifteen microliters of silica were added to the samples and gently shaked for 20min at room temperature. After centrifugation at 12,000×g for 1 min, the pellet was rinsed with washing buffer L2 (5.25 M GuSCN, 50 mM Tris-HCl, pH 6.4) and centrifuged at the same conditions. The pellet was first washed with 500µl of 70% ethanol, centrifuged again and rewashed with 500µl of acetone. The resulting pellet was air-dried in a water bath at 56°C for 15min and suspended in 60µL of MilliQ (for DNA) or DEPC-treated (for RNA) water, gently homogenized and incubated in a water bath at 56°C for 10min. After centrifugation at 12.000×g for 3min, 45 µL of the supernatant was collected and stored at -80°C until PCR analysis.

Extraction with FTA® Cards

Nucleic acids purification with Flinders Technology Associates (FTA®) filter paper cards was performed according manufacturer's instructions with some modifications. In the FTA card based protocol, seeded samples without previous treatment (10µl) were spotted directly onto filter circles (Whatman BioScience, Newtown Center, MA, USA) followed by air drying for 1h. As suggested by Bhattacharya et al. (2004), the DNA/RNA bound to the filter were then eluted with 400µl of a buffer containing 10mM Tris HCl pH 8.0, 0,1mM EDTA and 10mM β-mercaptoethanol, by intermittent vortex mixing for 15min. The filter paper was removed and DNA/RNA precipitated by the addition of one volume of 100% isopropanol for 10 min at RT. The resulting pellet was washed with 70% ethanol, dried at room temperature and suspended in 20µL of sterile MilliQ (for DNA) or DEPC-treated (for RNA) water and stored at -80°C until PCR analysis.

PCR for Advs Detection in Water and Oyster Samples:

Advs DNA was detected in water and oyster samples by using the oligonucleotide primers pair hexAA 1885 and hexAA 1913, which amplifies a 300bp-fragment from the Advs *hexon* gene (Allard et al., 1990; Girones et al., 1995, Rigotto et al., 2005). Five microliters of viral DNA and 30 pmoles of each primer were added to a reaction mix consisting of 20mM Tris-Cl, pH 8.4, 50mM KCl, 0.2mM each dATP, dCTP, dTTP and dGTP, 1.5mM $MgCl_2$ and 1U of Taq DNA polymerase (all reagents were purchased from Invitrogen Life Technologies, Carlsbad, CA,USA), in a final volume of 50µl. PCR amplification was carried out for 40 cycles of 94°C for 1min, 56°C for 1min and 72°C for 45s, with a final extension for 7min at 72°C. Negative (sterile MilliQ water) and positive controls (purified Advs DNA) were used in all experiments.

Reverse Transcription-PCR to Detect HAV in Water and Oyster Samples

HAV RNA was detected in water and oyster samples by reverse transcription PCR, using the oligonucleotide primers pair HAV-L and HAV-R, which amplifies a 192-bp amplicon from the VP1-VP3 protein capsid junction (Schwab et al., 1992, Sincero et al., 2006).

For cDNA synthesis, a 5.0µl aliquot of undiluted RNA was heated at 99°C for 5 min, followed by quick chilling on ice for 2min. The denatured RNA was added to a mixture containing 30 pmol of HAV-R, 50mM Tris-Cl, pH 8.4, 75mM KCl, 0.5mM of each dATP, dCTP, dTTP and dGTP, 2.5mM $MgCl_2$, 10mM dithiothreitol (DTT), 40U of RNAse inhibitor and 200U of M-MLV reverse transcriptase (all reagents were purchased from Invitrogen Life Technologies, Carlsbad, CA, USA), in a 25µL volume. Reverse transcription of viral genomic RNA was carried out at 37°C for 90min. Five microliters of cDNA and 30 pmol of each primer (HAV-L and HAV-R) were added to a reaction mixture consisting of 20mM Tris-Cl, pH 8.4, 50mM KCl, 0.2mM of each dATP, dCTP, dTTP and dGTP, 2.5mM $MgCl_2$ and 1U of Taq DNA polymerase (all reagents were purchased from Invitrogen Life Technologies), in a final volume of 50µL. PCR amplification was performed for an initial denaturation step of 95°C for 2min, followed by 40 cycles of 95°C for 1 min, 55°C for 1min and 72°C for 1 min, and a final incubation at 72°C for 7min. Negative (DEPC-treated water) and positive controls (purified HAV RNA) were used in all experiments.

Amplified fragments of both Advs and HAV seeded water and oyster samples were visualized by standard gel electrophoresis of 10µL of final reaction mixture in 1% agarose gels stained with ethidium bromide 1µg/mL.

RESULT

According to Table 1 and Figures 1 and 2, the detection limit for Adv using the positive control (infected cultured cells) was 3.5 PFU when organic-based extraction was used, 0.007 PFU for FTA® (Figure 1A) and 1.0 PFU for silica. In water samples, the detection limits for Adv were 1.4 PFU (organic based extraction) (Figure 1B), 3.5 PFU (FTA) and 45 PFU (silica) (Figure 1C). In oysters, the silica method could not detect adenovirus and the detection limit for phenol and FTA was 3,300 PFU and 1,400 PFU, respectively. For HAV-infected cells, the organic-based extraction detected 2,600 FFU, and either FTA or silica could detect 0.13 FFU. In water samples, the detection limits for HAV were 330 FFU (Trizol®) (Figure 2A), 1,000 (silica) (Figure 2B) and 160 (FTA). In oysters, the FTA method was not able to detect the hepatitis virus and the detection limits for organic-based extraction and silica method were both 6,600 PFU (Figure 2C).

Table 1: Detection limits of Adv and HAV viral particles from cell infected supernatant, water and oyster with three different methods of acid nucleic extraction: phenol/trizol, silica and FTA® card.

Sample	Infected cultured cells			Distillated water			Oyster extracts		
Method	Phenol/ Trizol	silica	FTA	Phenol/ trizol	Silica	FTA	Phenol/ trizol	silica	FTA
Advs (PFU)	3.5	1.0	0.007	1.4	45	3.5	3,300	negative	1,400
HAV (FFU)	2,600	0.13	0.13	330	1,000	160	6,600	6,600	negative

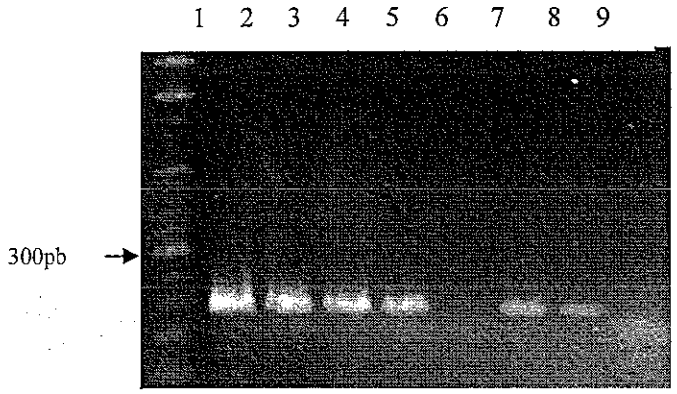

Figure 1A: Detection of adenovirus from infected cultured cells by FTA method. Values in PFU. (1)100pb ladder; (2) positive control; (3) 700; (4) 70; (5) 7; (6) 0.7; (7) 0.07; (8) 0.007; (9) negative control.

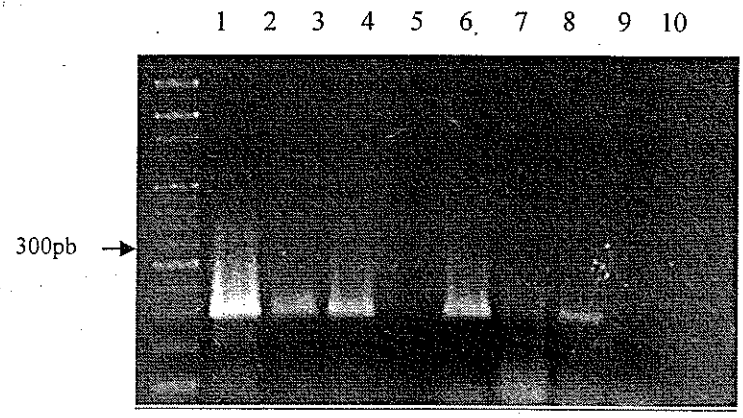

Figure 1B: Detection of adenovirus from distilled water by organic-based method. Values in PFU. (1)100pb ladder; (2) positive control; (3) 140,000; (4) 14,000; (5) 1,400; (6) 140; (7) 14; (8) 1.4, (9) 0.14; (10)negative control.

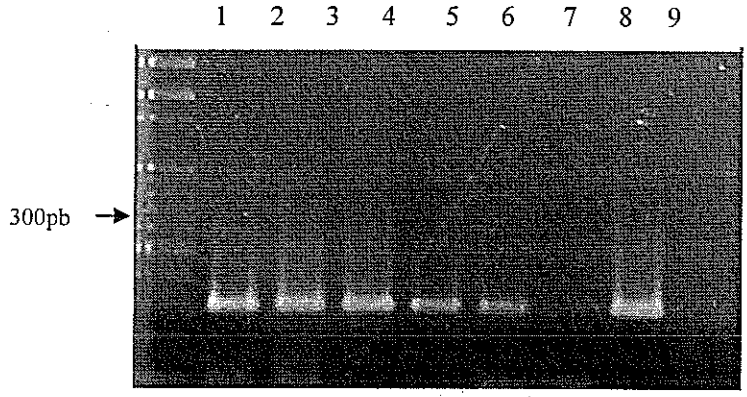

Figure 1C: Detection of adenovirus from infected cultured cells by silica method. Values in PFU. (1)100pb ladder; (2) 10,000; (3) 1000; (4) 100; (5) 10; (6) 1.0; (7) 0.1; (8) positive control; (9) negative control.

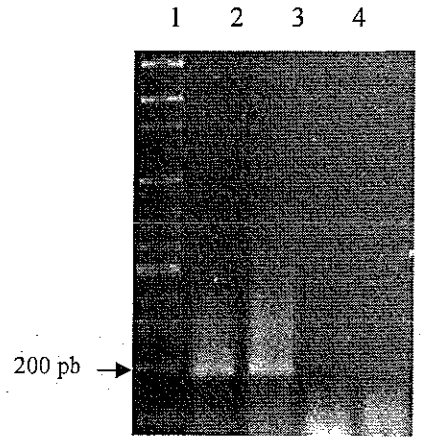

Figure 2A: Detection of HAV from distilled water by organic-based method. Values in FFU. (1)100pb ladder; (2) 3,300; (3) 330; (4) 33; (5) negative control.

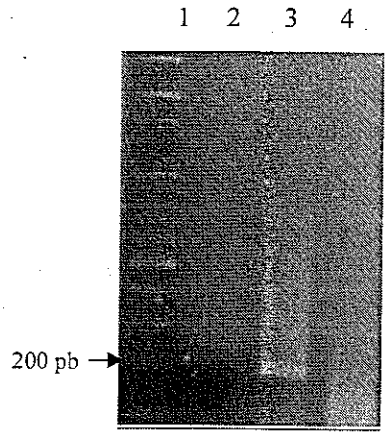

Figure 2B: Detection of HAV from distilled water by silica method. Values in FFU. (1)100pb ladder; (2) negative control; (3) 1000; (4) 100.

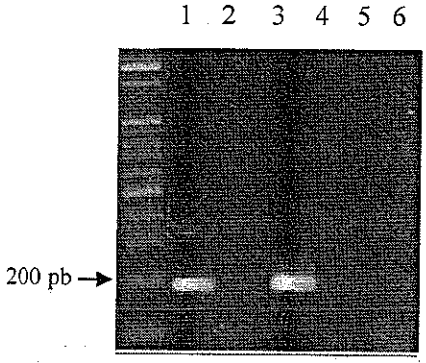

Figure 2C: Detection of HAV from oysters by organic-based and silica methods. Values in FFU. (1)100pb ladder; organic-based method: (2) 6,600 and (3) 660; silica method: (4) 6,600 and (5) 660; (6) negative control.

DISCUSSION

In the present study, we have demonstrated a comparison of three different nucleic acid extraction methods of viruses seeded from several samples (i.e. oyster tissues, distillated water and infected cultured cells). These methods aimed to extract and purify nucleic acids in order to remove cell debris and inhibitors before amplification and detection by PCR (Griffin et al., 2001; Lipp, et al., 2001). One of the most widely used methods for viral nucleic acid extraction and purification has been developed by Boom et al. (1990) and is based on guanidium thiocyanate (GuSCN) extraction and use of silica columns to bind and wash nucleic acids. This method is rapid, easy to use and efficient in removing inhibitors (Jiang at al., 2001). In addition, it diminishes cross contamination between samples and it is an efficient method for pathogen recovery in environmental samples. Moreover, it has been shown to be an excellent method for detection of Advs, HAV, Enterovirus, Polyoma viruses

and Hepatitis E viruses recovered from drinking water, river water, seawater and sewage (Puig et al., 1994; Pina et al., 1998; Bofill-Mas et al., 2006).

We have found that, when compared with organic-based extraction methods, the silica method showed the best results for viral detection (3.5 PFU Advs and 2,600 FFU HAV vs 1.0 PFU Advs and 0.13 FFU HAV) from *in vitro* infected cells. However, the silica method was less efficient than the FTA-based protocol (0.007 PFU for Adv and 0.13 FFU for HAV). While in water samples, the silica-based protocol showed the highest detection limits for Advs (45 PFU), in oysters, the same method was not able to recover DNA from Advs and displayed a low recovery limit for HAV (6,600 FFU). These results showed that a prior clarification procedure using proteinase K or CTAB is necessary, similar to what has been done inorganic extraction-based methods. Nevertheless, a prior treatment would make the method more laborious and time consuming. The silica is a simple and low cost method for nucleic acid extraction, and can be useful for a large number of samples.

According to current literature, FTA technology is suitable for microorganism recovery from many kinds of samples including blood, plant and animal tissues, stool and salive (Moscoso et al., 2004; Ndunguru et al., 2005). The handling time for the FTA extraction-free procedure is low and includes only few washes of the bound acid nucleic, as opposed to the lengthy phenol/chloroform extraction procedures (Moscoso et al., 2004). Thus FTA was the best method to recover and detect up to 0.007 PFU for Advs, and 0.13 FFU for HAV from in vitro virus-infected cells, while the organic-based and silica extraction methods detected 3.5 PFU and 1.0 PFU, for Advs and 2,600 FFU and 0.13 FFU, for HAV. The FTA filter was efficient to recover HAV from water samples up to 160 FFU, 3.5 PFU of Adenovirus and failed to detect HAV from oyster samples. These results demonstrate that the environmental samples are highly heterogeneous and variation of virus content as well as PCR inhibitors present in the samples make comparison difficult. Several studies have reported that the FTA card can be applied to both DNA and RNA viruses, with better results when compared to conventional isolation methods (Smith and Burgoyne, 2004; Desloire et al., 2006). According to Ndunguru et al. (2005), RNA and low molecular weight DNA molecules, such as plasmids and viral genomic components, are eluted by a single extraction buffer and used as template for amplification by PCR. The genomic DNA remains attached to the paper matrix but is available for amplification by PCR when the paper punch is included in the PCR reaction mix. In our work, both DNA and RNA were eluted from FTA card by an extraction buffer and probably, the nucleic acids recovery was not complete.

Advantages of FTA technology have been proposed for human DNA processing and forensic applications, for wildlife DNA samples and applied to PCR-based genotyping (Dan-Myet al., 2004; Desloire et al., 2006; Nantavisai et al., 2007), but have not been well documented for use with water and food samples to detect viral pathogens. We suggest the use of FTA for clean samples, like infected cultured cells, because when more complex samples are used, such as water and oysters, it would be necessary more clarification steps before extraction, like we suggested for silica method. We used the treatment with proteinase K and CTAB to purify and remove inhibitors from oyster samples before the organic-based extraction. Other methods for viral nucleic acid extraction and purification include proteinase K treatment followed by phenol-chloroform extraction and ethanol precipitation, sonication and heat treatment (Albert and Schwartzbrod, 1991; Le Guyader et al., 1994; Bosch et al., 1997; Chapron et al., 2000; Monpoeho et al., 2001).

Casas et al. (1995) developed an extraction method using GuSCN and an inorganic solvent to purify both viral RNA and DNA in a single extraction step. Extraction kits based on modifications of these methods are commercially available (Griffin et al., 1999; Jiang et al., 2001; Lipp et al., 2001), but they are expensive and many routine analysis laboratories will not be able to afford them. The organic-based extraction method for virus-infected cells, like the other methods showed a satisfactory viral recovery (3.5 PFU for Advs and 2,600 for HAV). For concentrated oyster samples the organic-based extraction methods demonstrated better recovery (3,300 PFU for Advs and 6,600 FFU for HAVs) than the others, probably because it was employed previous clarification steps to remove inhibitors. For large amounts of samples like in routine and environmental analysis, it becomes very laborious and time consuming. For water samples this method had a better recovery yield for Advs (1.4 PFU) when compared with HAV (330 FFU). This can be due to the nature of the virus genomes (DNA for Advs and RNA for HAV), the virus stability in the samples but this has to be confirmed by further studies involving other environmental samples as seawater, wastewater and tap water. To improve the sensitivity of the detection methods another molecular associated protocols, as Real-time-PCR, can be associated.

CONCLUSION

Silica method can be considered as a golden method for virus detection in water samples especially when a large number of samples have to be analyzed.

The organic-based extraction methods are more suitable for virus detection in mollusk samples.

FTA method is very useful to extract virus genomes from infected cultured cells. In conclusion, this research field must be more explored to increase methods sensitivity and to facilitate virus detection in environmental samples.

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