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LEONARDO DINIZ MENDES

TORQUE TENO VIRUS: DIVERSIDADE GENÉTICA E AVALIAÇÃO, NA BACIA AMAZÔNICA, DO SEU POTENCIAL COMO MARCADOR DE POLUIÇÃO ANTRÓPICA

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ORIENTADORES: Dr. Christian Maurice Gabriel Niel
Dra. Marize Pereira Miagostovich

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ORIENTADORES: **Dr. Christian Maurice Gabriel Niel**
Laboratório de Virologia Molecular - IOC

Dra. Marize Pereira Miagostovich
Laboratório de Virologia Comparada - IOC

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Dr. Eduardo de Mello Volotão - IOC/FIOCRUZ

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Água
Arnaldo Antunes e Paulo Tatit

Da nuvem até o chão
Do chão até o bueiro
Do bueiro até o cano
Do cano até o rio
Do rio até a cachoeira
Da cachoeira até a represa
Da represa até a caixa d´água
Da caixa d´água até a torneira
Da torneira até o filtro
Do filtro até o copo
Do copo até a boca
Da boca até a bexiga
Da bexiga até a privada
Da privada até o cano
Do cano até o rio
Do rio até outro rio
Do outro rio até o mar
Do mar até outra nuvem

Torque teno virus: Diversidade genética e avaliação, na bacia amazônica, do seu potencial como marcador de poluição antrópica

RESUMO

O torque teno virus (TTV) é um vírus DNA, com genoma circular de fita simples com grande variabilidade genética apresentando caráter ubiqüitário, altíssima prevalência em indivíduos sadios ou não, viremia constante e independente de surtos ou sazonalidade, resistência e distribuição global. A presença do TTV no ambiente se configura como uma fonte potencial de informação indicadora universal da presença de contaminação gerada pela atividade humana. Os objetivos deste estudo foram avaliar a diversidade genética e o potencial do TTV como indicador de contaminação de natureza antrópica, na bacia Amazônica. Neste estudo, a diversidade genética do TTV foi investigada, tendo sido possível a descrição de dois novos genótipos de TTV humano e de uma cepa de TTV suíno, denominada Sd-TTV2p, que passa a ser o protótipo de um novo genogrupo (genogrupo 2). Para avaliar a disseminação dos vírus entéricos no ambiente, dois protocolos de concentração viral foram testados utilizando o vírus da hepatite A (HAV) como modelo. O protocolo de concentração de partículas virais utilizando membrana carregada negativamente, modificado pela adição de íons magnésio, e com reconcentração por ultrafiltração, se mostrou eficaz para a concentração de HAV em amostras de água de rio. Esta metodologia foi então empregada na concentração de partículas virais em amostras de água de igarapés da bacia Amazônica em Manaus para o monitoramento da presença dos vírus TTV, HAV, adenovirus, rotavirus, norovirus e astrovirus. Também foi desenvolvido um protocolo para detecção molecular quantitativa pela reação em cadeia da polimerase (qPCR) para determinar a concentração do TTV na área estudada. A utilização da qPCR para a detecção de TTV no ambiente elevou o percentual de amostras positivas para 92% quando comparado com os resultados obtidos por PCR qualitativo (37%). O genoma do TTV pôde ser detectado em concentrações que variaram de 10^3 a 10^5 genomas-equivalente (gEq)/100 mL de água coletada, demonstrando a elevada concentração deste vírus nos igarapés. A PCR qualitativa, embora menos sensível que a qPCR, foi importante para a avaliação da diversidade genética do TTV, já que o sequenciamento nucleotídico dos produtos de PCR confirmou a origem humana do vírus. Uma grande diversidade genética do TTV foi observada nas amostras ambientais. Dentre as 19 amostras que puderam ser amplificadas, 11 seqüências distintas puderam ser observadas. A presença do TTV não apresentou correlação significativa com a presença de outros patógenos nem com os demais indicadores físico-químicos. Contudo, a não correlação do TTV com os outros parâmetros de qualidade pode representar uma característica importante que contribui para o estabelecimento do TTV como um indicador sensível e universal da qualidade da água. A análise comparativa dos resultados obtidos neste estudo reforça o potencial do TTV humano como indicador de poluição antrópica. No entanto, novos estudos serão necessários para determinar sua utilização como um marcador virológico de potabilidade da água.

Ministério da Saúde
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INSTITUTO OSWALDO CRUZ

Doctoral Thesis
Leonardo Diniz Mendes

Torque teno virus: Genetic diversity and evaluation, in the Amazon basin, of its potential role as indicator of anthropic pollution

ABSTRACT

Torque teno virus (TTV) is a ubiquitous single-strand DNA virus with a great genetic diversity and a very high prevalence even among healthy individuals. TTV is globally spread and its infection is persistent and independent from outbreaks and seasonality. TTV presence in the environment may constitute important information to reveal water contamination from human origin. The aims of this study are to evaluate the genetic variability and the potential of TTV as an indicator of anthropic pollution, in the Amazon basin. TTV genetic diversity was observed in human samples with the description of two new TTV genotypes. A new swine TTV genomic group was also described and called TTV genogroup 2. Two protocols for virus concentration, using hepatitis A virus (HAV) as a model, were tested for the evaluation of dissemination of enteric viruses. The method, based on virus particles adsorption to negatively charged filters, with addition of magnesium chloride and ultrafitration re-concentration, was suitable for virus recovery from river water. This protocol was then used to concentrate viruses from water samples collected from small rivers in the city of Manaus. The presence of the TTV, HAV, adenovirus, rotavirus, norovirus and astrovirus was evaluated. A quantitative polimerase chain reaction (qPCR) assay was developed for TTV detection in the studied area. qPCR showed to be highly sensitive, raising the TTV prevalence from 37%, obtained by conventional PCR, to 92%. TTV genome was detected in levels ranging from 10^3 a 10^5 genome-equivalent (gEq)/100 mL of water, revealing the high concentration of this virus in the samples analyzed. Qualitative PCR was an important tool for the confirmation of the human origin of the amplicons by nucleotide sequencing. The high TTV genetic diversity was also observed within the environment samples. From 19 samples, 11 distinct sequences were depicted. TTV presence was significantly correlated neither to the presence of other pathogens nor to the levels of physico-chemical pollution indicators. However, that absence of correlation may be considered as a positive feature for the establishment of TTV as a sensitive and universal indicator of water quality. The comparative analysis of the results generated in this work reinforces the potential of human TTV as an indicator of anthropic pollution. However, further studies are still necessary to determine its application as a viral indicator of water quality.

LISTA DE ABREVIATURAS

AdV - adenovírus
AM – Estado do Amazonas
AstV - astrovirus
DNA – ácido desoxirribonucléico
EV - enterovírus
gEq - genoma-equivalente
HAV - vírus da hepatite A
HEV - vírus da hepatite E
ICTV - Comitê Internacional de Taxonomia de Vírus
L – litro
M – molar
nm – nanômetro
NoV - norovírus
ORF - fase aberta de leitura
pb – pares de base
PCR - reação em cadeia da polimerase
qPCR - reação em cadeia da polimerase quantitativa
RCA - amplificação em círculo rolante
RNA - ácido ribonucléico
RT-PCR - reação em cadeia pela polimerase após transcrição reversa
RV - rotavírus
TTMDV – torque teno midivirus
TTMV – torque teno minivirus
TTV – torque teno vírus
USEPA - Agência de Proteção Ambiental dos Estados Unidos

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1. INTRODUÇÃO

1.1. Torque teno virus

1.1.1. Histórico e classificação

Durante uma pesquisa sobre vírus ainda desconhecidos, um novo vírus foi detectado pela primeira vez em um paciente japonês, vítima de hepatite pós-transfusional, de etiologia deconhecida (Nishizawa et al. 1997). O paciente de 58 anos havia sido submetido a uma cirurgia cardíaca onde recebeu transfusão sanguínea de 35 doadores diferentes. Na décima semana após a cirurgia, seus níveis séricos da enzima hepática alanina aminotransferase alcançaram um pico de 180 UI/L. O soro do paciente foi coletado durante todo o curso da doença e submetido à técnica chamada *Representational Difference Analysis* (RDA) (Lisitsyn et al. 1993). Esta técnica, que permite a amplificação inespecífica do material genético presente em uma dada amostra, possibilitou a detecção transitória de determinadas sequências de ácidos nucléicos durante a fase aguda da doença. A análise da seqüência obtida do primeiro clone (N22) contendo um inserto de 500 pares de base (pb), não revelou identidade significativa com nenhuma das 1,7 milhão de seqüências disponíveis nos bancos de dados em outubro de 1997. Devido às iniciais do paciente serem T.T., o recém descoberto vírus foi inicialmente designado como vírus TT e recebeu a sigla em inglês TTV (Nishizawa et al. 1997).

A observação de que o material não foi amplificado em amostras de soro de outros humanos demonstrou a natureza exógena do material previamente detectado (Okamoto et al. 1998a). Este estudo revelou ainda outras características do material: (i) densidade de 1,26 g/cm^3 em gradiente de sacarose; (ii) resistência a desoxiribonuclease (DNase) I que levou a conclusão de que era encapsidado, tratando-se portanto de um vírus; (iii) resistência a enzimas de restrição e ribonuclease (RNase) A e sensibilidade a nuclease de feijão Mungo demonstrou que se tratava de um DNA de fita simples; (iv) a densidade não alterada após tratamento com detergentes sugeriu que a partícula viral não era envelopada (Okamoto et al. 1998a).

No ano seguinte, foi descrita a presença de uma região de 120 nucleotídeos, rica em guanina (G) e citosina (C), que ligava as extremidades 3’ e 5’ dos genomas já sequenciados até então levando a conclusão de que a conformação do genoma do TTV é circular e de polaridade negativa (Miyata et al. 1999; Mushawar et al. 1999). Em seguida, o primeiro genoma completo de um TTV foi sequenciado. O clone então denominado TA278 possui genoma com tamanho de 3.853 nucleotídeos e, estruturas específicas na região rica em G e C que podem desempenhar papéis importantes na replicação viral (Okamoto et al. 1999 c,d).

Atualmente, por determinação do Comitê Internacional de Taxonomia de Vírus (ICTV), a sigla TTV é atribuída ao nome torque teno virus, do latim *torques* (que significa colar) e *tenuis* (que significa fino), referindo-se ao genoma de DNA circular de fita-simples (Biagini et al. 2004). Apesar de inicialmente ter sido relacionado com o quadro clínico de hepatite, a capacidade do TTV em induzir esta ou qualquer outra enfermidade ainda se encontra em discussão.

Uma grande divergência entre as seqüências de nucleotídeos e aminoácidos dos diferentes genótipos descritos de TTV foi observada após sua descrição (Okamoto et al. 1998a). As divergências entre as seqüências de aminoácidos encontradas variam entre 47 e 70% (Biagini et al. 1999; Luo et al. 2002). A análise das seqüências de nucleotídeos de TTV classifica os 61 genótipos já descritos em cinco grupos filogenéticos (Biagini et al. 2004) (Figura 1).

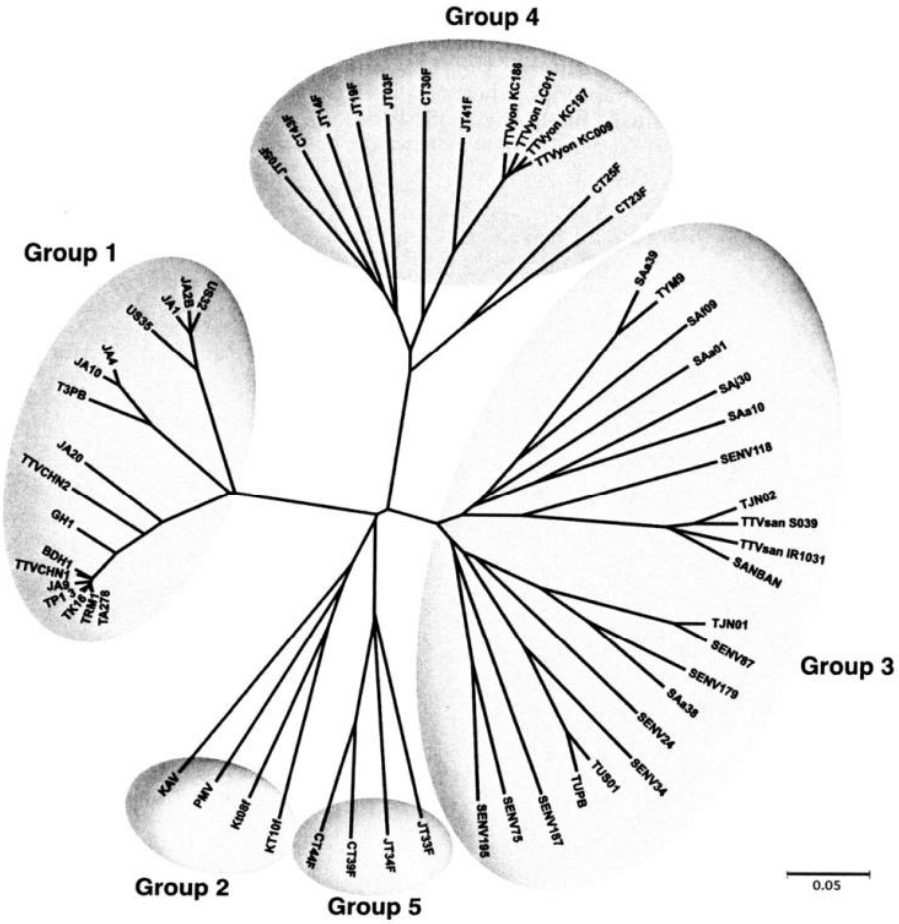

Figura 1. Árvore filogenética dos torque teno virus. A barra representa uma distancia genética de 5% (Biagini et al. 2004).

O TTV também foi encontrado em várias espécies animais mostrando não ser restrito apenas aos humanos. Estes vírus espécie-específicos apresentam similaridades com a organização genômica do TTV humano e infectam primatas não humanos, porcos, gatos e cães (Leary et al. 1999; Verschoor et al. 1999; Okamoto et al. 2002).

O TTV compartilha similaridades na organização do genoma, diversidade genética e prevalência em humanos, com outros dois vírus: o torque teno minivírus (TTMV) (Takahashi et al. 2000) e o torque teno midivírus (TTMDV) (Ninomiya et al. 2007). A análise das seqüências da região da fase aberta de leitura (ORF) 1 dos três vírus demonstra que eles formam grupos filogenéticos distintos (Figura 2).

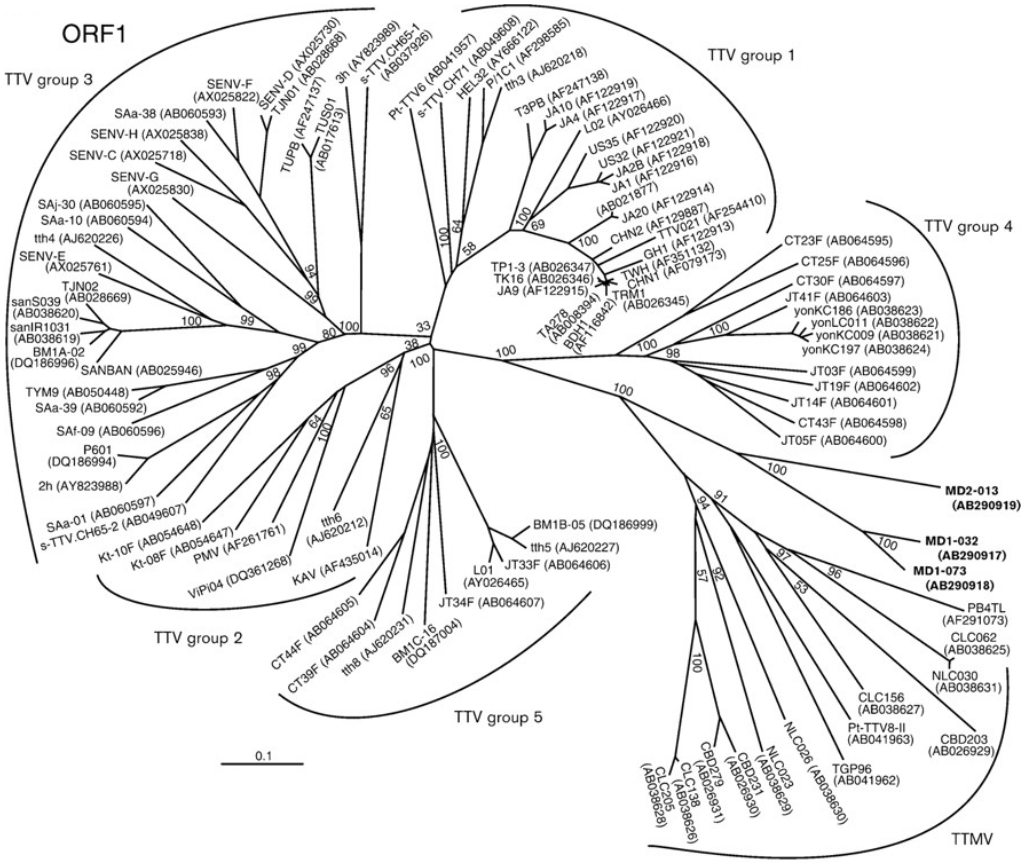

Figura 2. Árvore filogenética construída com base na seqüência da fase aberta de leitura 1 dos três anelovírus humanos, torque teno virus (TTV), torque teno minivírus (TTMV) e torque teno midivírus TTMDV (genótipos com nomes iniciados por MD) (Ninomiya et al. 2007).

Atualmente, o TTV e os seus similares estão classificados no gênero flutuante *Anellovirus* (Biagini et al. 2004), sendo proposto pelo grupo de estudos de *Circoviridae-Anellovirus* do ICTV a criação de uma nova família chamada *Anelloviridae* para classificação dos três representantes vírus de humanos (TTV, TTMV e TTMDV) e os que infectam animais (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/00.107.0.01.001.htm).

1.1.2. Morfologia, genoma e proteínas virais

O TTV é um vírus não-envelopado com partículas de diâmetro variando de 30 a 50 nm (Okamoto et al. 1998). O genoma circular de DNA fita-simples, polaridade negativa (Mushahwar et al. 1999), possui tamanho de 3,8-3,9 kb de acordo com os diferentes genótipos (Miyata et al. 1999;

Peng et al. 2002) e caracterizado por uma região codificante contendo seis ORFs e uma região não-codificante conservado. A região não-codificante de 1,2 kb é marcada pela presença de uma região rica em citosinas e guaninas presente em todos os genótipos (Peng et at. 2002) e uma região promotora *TATA box* (Hallett et al. 2000). O produtos destas ORFs, apesar de ainda estarem em estudo, parecem estar envolvidos na estrutura do capsídeo (Takahashi et al. 1998), na replicação (Erker et al. 1999) e regulação do ciclo celular (Asabe et al. 2001) e da transcrição (Kamahora et al. 2000)(Figura 3).

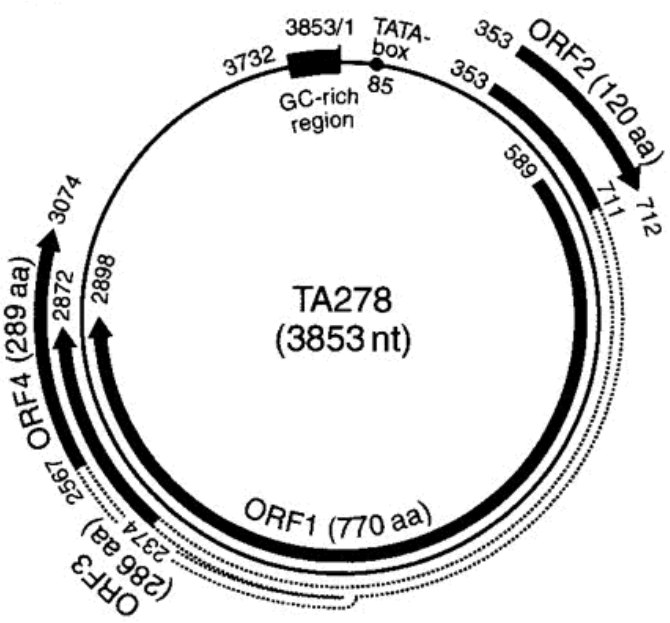

Figura 3. Organização do genoma do torque teno virus (Okamoto et al. 2001b).

A região codificante que apresenta tamanho de aproximadamente 2,6 kb, apresenta quatro ORFs sobrepostas (Miyata et al. 1999). Três RNAs-mensageiro (RNAm) com tamanhos de 2,8 kb, 1,2 kb e 1,0 kb são formados por um processo conhecido como processamento alternativo do RNA (do inglês "*alternative splicing")* a partir da região compreendida entre os nucleotídeos 109 e 3006 do da cepa TYM9 (Okamoto et al. 2000b). Um estudo recente utilizando como modelo a cepa HEL32, sugere que estes três RNAm dão origem a seis fases de leitura aberta (ORFs) que codificam

proteínas hipotéticas de aproximadamente 736 aminoácidos (aa) [ORF1], 117 aa [ORF2], 281 aa [ORF3], 275 aa [ORF4], 195 aa [ORF5] e 142 aa [ORF6] (Qiu et al. 2005) (Figura 4).

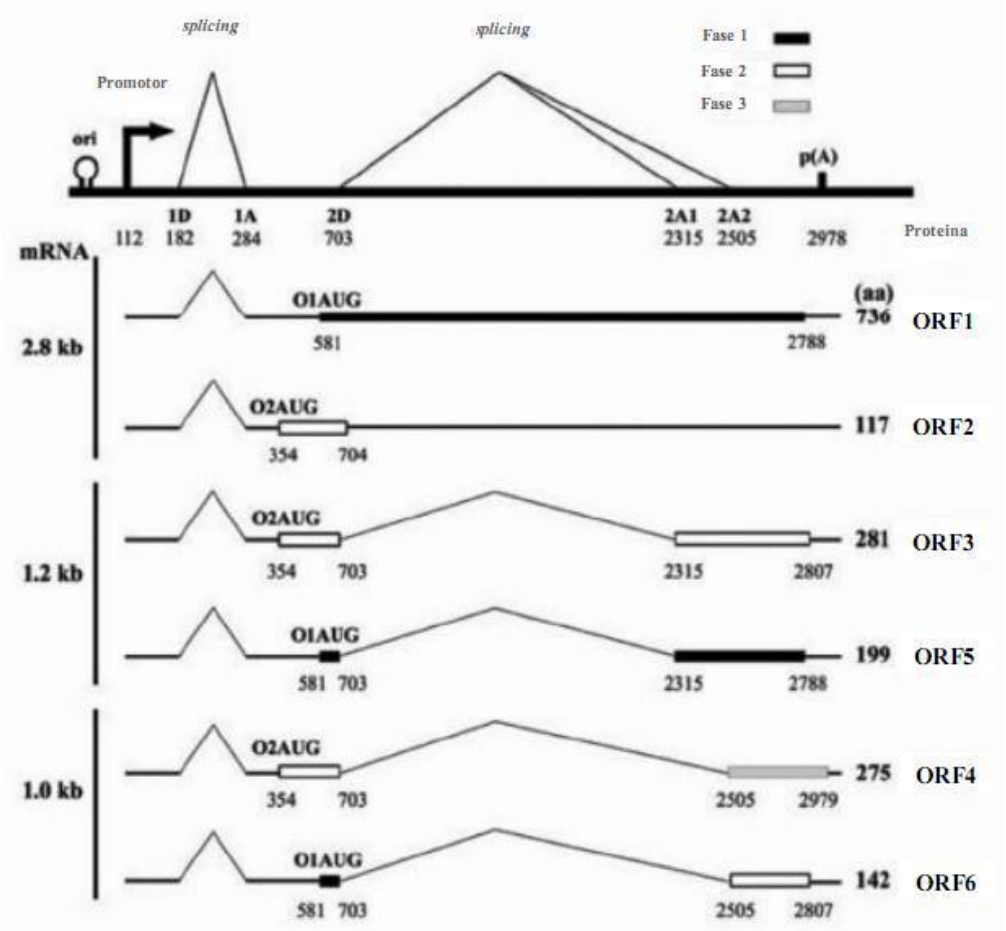

Figura 4. Localização das seis ORFs do TTV no genoma do isolado HEL32. Adaptado de Qiu et al., 2005.

A partir do RNAm de 2,8 kb são traduzidas as ORFs 1, que codifica uma proteína estrutural, responsável pela formação do capsídeo viral (Hallett et al. 2000), e a ORF 2 que parece produzir uma proteína que atua na defesa viral (Zheng et al. 2007) contra o ataque do sistema imunológico do hospedeiro. Estas duas ORFs são oriundas de transcrição em duas fases diferentes, fase 1 e 2, respectivamente, e não sofrem processamento posterior. O RNAm de 1,2 kb codifica outras duas proteinas: (i) a ORF 3 a qual acredita-se que induza a apoptose celular (Kooistra et al. 2004), essa

é formada pela junção das extremidades amino e carboxi-terminais que se encontram na fase 2, e (ii) a ORF 5, formada pela junção das extremidades amino e carboxi-terminais que se encontram na fase 1. As ORFs 4 e 6 têm sua origem a partir do RNAm de 1,0 kb. A proteína originada da ORF 4 é formada pela junção de uma porção amino-terminal traduzida de um segmento que se encontra na fase 2 e de uma região carboxi-terminal traduzida de um segmento oriundo da fase 3. Diferentemente, a ORF 6 codifica uma proteína formada pela junção de uma porção amino-terminal traduzida de um segmento da fase 1 e, de uma região carboxi-terminal traduzida de um segmento da fase 2 (Figura 4). As funções das ORFs 4, 5 e 6 ainda encontram-se sob estudo.

1.1.3. Diversidade genética do torque teno virus

Ao contrário da maioria dos vírus com genoma composto por DNA, os TTVs exibem um alto percentual de heterogeneidade entre as seqüências de nucleotídeos e de aminoácidos (Bendinelli et al. 2001). A comparação entre as seqüências disponíveis nos bancos de dados demonstra que a divergência não ocorre igualmente ao longo do genoma (Itoh et al. 1999). As seqüências de DNA da região não codificante possuem segmentos com até 90% de identidade entre os genótipos. Em contraste, a região transcrita apresenta regiões com apenas 30% de identidade (Biagini et al. 2006). As identidades observadas entre os cinco grandes grupos filogenéticos de TTV são menores que 60%, analisando-se o genoma completo. Com toda a variação observada, não é difícil de se imaginar que toda a extensão da diversidade do TTV, com a descrição de novos grupos genômicos e/ou genótipos, ainda não foi completamente relatada. Algumas razões podem sustentar essa premissa: (i) os protocolos de PCR utilizados na maioria dos estudos são deficientes na detecção de um ou mais grupos e/ou genótipos, não sendo capazes de detectar toda a variedade de TTVs, e (ii) existem indicativos de que os diferentes TTVs humanos possam ser capazes de se recombinarem enquanto co-infectam um mesmo indivíduo (Abe et al. 2000; Okamoto et al. 2000a; Romeo et al.

2000; Manni et al. 2002). A capacidade de produzir infecções crônicas com baixa viremia (Prescott et al. 1999), a possibilidade de infectarem espécies diferentes e a capacidade de recombinação, como ocorre em outros vírus de DNA fita-simples (Gibbs & Weiller, 1999), facilitam a expansão da diversidade genética do grupo.

1.1.4. Metodologia de detecção

O TTV não é propagado em cultivos celulares convencionais (Hino & Miyata, 2007). Por esta razão a principal forma de detecção deste organismo é a amplificação de fragmentos de seu material genético pela da reação em cadeia da polimerase (PCR). Devido à grande variabilidade genética torna-se difícil o estabelecimento de um ensaio de PCR com abrangência universal. Um grande número de protocolos descritos utiliza oligonucleotídeos iniciadores que hibridam em regiões conservadas da ORF1 (Okamoto et al. 1998b, Nishizawa et al. 1999) e na região não codificante (Takahashi et al. 1998; Okamoto et al. 1999; Biagini et al. 2001), onde se observa maior conservação do genoma. Dependendo das condições da PCR e dos oligonucleotídeos iniciadores utilizados algum tipo de interferência na sensibilidade do método pode ser observada (Bendinelli et al. 2001).

1.1.5. Epidemiologia

O TTV está disseminado mundialmente na população (Prescott & Simmonds, 1998; Viazov et al. 1998; Abe et al. 1999; Okamoto et al. 1999) e sua prevalência aumenta conforme a faixa etária (Hsieh et al. 1999; Saback et al. 1999; Umemura et al. 2001a; Zhong et al. 2001). A avaliação sobre a prevalência do TTV pode variar dependendo do método de PCR utilizado (Niel et al. 2005; Biagini et al. 2007). Em doadores de sangue, o TTV foi encontrado em 46-62%, no Brasil (Niel et al. 1999;

Devalle & Niel 2004), 51,6 a 82,7%, na Turquia (Erensoy et al. 2002; Yazici et al. 2002), 90% na Noruega (Huang et al. 2001) e 53,3% na China (Zhong et al. 2001).

A real representação do TTV nos humanos é um tópico que ainda se encontra em discussão. Contudo, é consensual que o TTV está presente no sangue da maioria dos indivíduos aparentemente saudáveis em todo o mundo. É eliminado em vários fluidos corporais como saliva, lágrimas, leite materno, sêmen, secreções vaginais (Deng et al. 2000; Inami et al. 2000; Matsubara et al. 2000; Calcaterra et al. 2001) e, principalmente, nas fezes (Okamoto et al. 1998a; Ross et al. 1999; Tawara et al. 2000; Okamoto et al. 2001b), sugerindo uma transmissão via fecal-oral. A excreção fecal pôde ser comprovada pela visualização partículas de TTV por microscopia eletrônica em amostras de fezes (Figura 5) (Itoh et al. 2000).

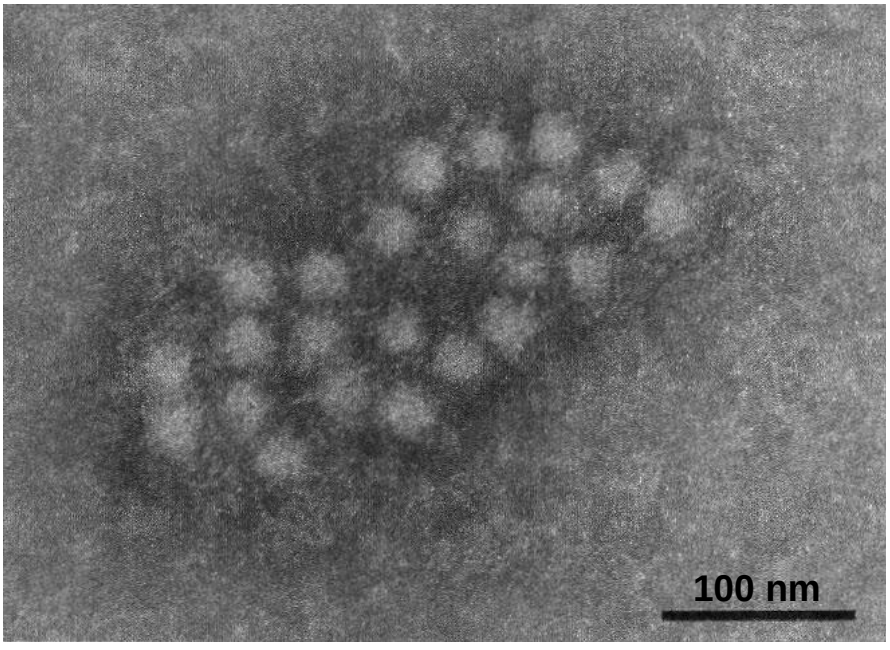

Figura 5. Microscopia eletrônica de torque teno virus. Barra de escala igual a 100 nm (Itoh et al. 2000).

O TTV é resistente à inativação por tratamento com solvente-detergente e aquecimento a 65°C/96 horas a seco (Simmonds et al. 1998; Chen et al. 1999). Esses dados, juntamente com a estrutura viral

conhecida, sugerem que o TTV possa ser tão estável quanto o parvovírus (Berns, 1996).

Como outros vírus entéricos, o TTV pode permanecer infeccioso durante meses no ambiente, resistindo à condições adversas. A presença do TTV tem sido descrita em vários ambientes aquáticos, sendo detectado em amostras de água de rio - 5% no Japão (Haramoto et al. 2005a) e 25% na Itália (Verani et al. 2006) – e em amostras de esgoto - 5% na Índia e no Japão (Vaidya et al. 2002; Haramoto et al. 2005b).

1.2. Virologia Ambiental

A escassez dos recursos hídricos e a poluição ambiental têm colocado a questão do uso e da gestão da água no centro dos interesses em todo o mundo. Embora o planeta disponha de água suficiente para toda a população, estima-se que mais de 1 bilhão (18%) de pessoas continuem sem acesso a água potável e que 2,6 bilhões (42%) não disponham de tratamento de água (Nações Unidas, Relatório sobre Desenvolvimento Humano, 2006).

Dentre os maiores problemas da utilização da água, se destaca a sua poluição por resíduos biológicos, considerando o crescimento populacional e o aumento do consumo. Águas contaminadas são um importante meio de transmissão de doenças, sendo o despejo de esgoto sem tratamento o mais freqüente fator de contaminação. Doenças veiculadas pela água constituem uma das causas comuns de morte no mundo e afetam especialmente países em desenvolvimento. Estima-se que 25% dos leitos hospitalares sejam ocupados por pacientes com doenças transmitidas por veiculação hídrica (Straub & Chandler, 2003).

Uma variedade de vírus como rotavírus (RV), norovírus (NoV), adenovírus (AdV), astrovirus (AstV), enterovírus (EV), os vírus da hepatite A (HAV) e E (HEV), e TTV presentes no trato gastrintestinal de indivíduos infectados, são eliminados através das fezes em grandes quantidades (10^5-

10^{11}/g de fezes) no meio ambiente (Bosch, 1998; Okamoto et al. 1998). Dispersos na água, podem alcançar áreas de mananciais e recreacionais (Figura 6) devido à contaminação por excretas, como resultado de conexões cruzadas e problemas de manutenção na rede de distribuição de água e coleta de esgoto e saneamento básico deficiente.

Os vírus podem ser transmitidos pelo o consumo de água ou alimentos contaminados, pelo contato direto ou por ingestão acidental (Bosch, 1998; Wyn-Jones & Sellwood, 2001).

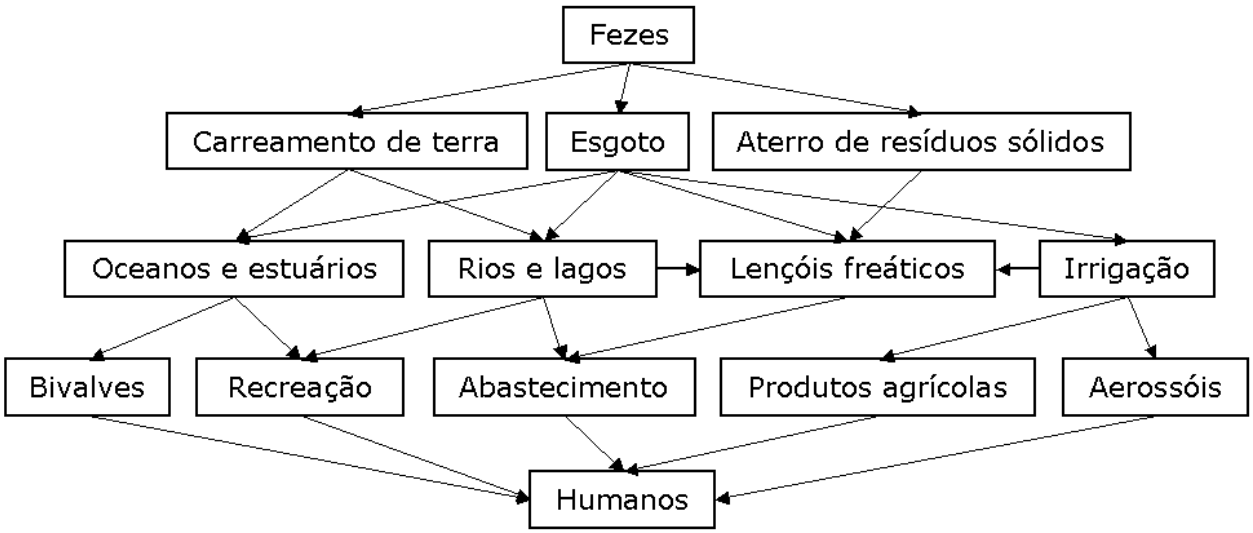

Figura 6. Rotas de transmissão de vírus entéricos no ambiente (adaptado de Metcalf et al. 1995).

Os primeiros estudos em virologia ambiental tiveram início na década de 1940 com a tentativa de se detectar poliovírus pela inoculação de águas de esgoto em macacos. Esse estudo demonstrou que quando os casos de paralisia eram prevalentes na comunidade, os poliovírus estavam presentes no esgoto (Melnick, 1947).

Na década de 1950, a ocorrência de um surto de hepatite em Nova Delhi, na Índia, ocasionado pela contaminação do sistema de tratamento da água por esgoto, resultou no registro de 30 mil casos. Posteriormente, o HEV foi identificado como o agente etiológico desta epidemia,

considerada como uma das maiores de veiculação hídrica já descritas (Bosch, 1998).

Este tema despertou interesse da comunidade ao longo dos anos e, em 1965, foi realizada a primeira conferência internacional denominada ("Transmissão de vírus pela da água") na cidade de Cincinnati, Ohio, EUA. Outras conferências foram realizadas nas décadas seguintes no México ("Vírus na Água" - 1974) e em Israel ("Vírus Entéricos na Água" - 1982). Temas como desenvolvimento de métodos capazes de detectar baixas concentrações de vírus em ambiente aquático, correlação de parâmetros biológicos com a manutenção dos vírus em água, determinação de dose infectante de cada tipo de vírus, persistência dos vírus nas águas de reuso e a importância de se estabelecer um padrão viral de qualidade da água foram discutidos. A partir de então, diferentes estudos foram desenvolvidos contribuindo para o desenvolvimento da virologia ambiental a partir da década de 1980 (Metcalf et al. 1995).

1.3. Vírus como marcadores de poluição antrópica

Os métodos de monitoramento de qualidade da água de recreação e consumo, utilizados atualmente, são baseados em marcadores físico-químicos (e.g. pH, condutividade, turbidez, temperatura, oxigênio dissolvido, etc.) e bacterianos (coliformes totais e fecais). Contudo, os indicadores bacteriológicos clássicos, coliformes termotolerantes e *Escherichia coli* não são efetivos para avaliar a ocorrência e a remoção de vírus da água com coliformes fecais. Estudos comparativos da detecção de EV, AdV, RV, AstV, HAV e NoV (Schvoerer et al. 2000; Gofti-Laroche et al. 2001; Kittigul et al. 2001; Schvoerer et al. 2001; Lee & Kim, 2002; Borchardt et al. 2003) demonstraram que não há correlação entre estes agentes nas amostras de água examinadas: água de esgotos, rios, córregos, poços e torneiras.

A ausência de informação sobre a presença de vírus na água torna incompleta a estimativa da condição de balneabilidade de determinada área. Uma vez que os vírus entéricos são mais resistentes à degradação do

que as bactérias fecais (Shuval, 1971), eles podem ser detectados até em águas dentro dos padrões de qualidade (Griffin et al. 1999; Noble & Fuhrman, 2001).

A Agência de Proteção Ambiental dos Estados Unidos (*United States Environmental Protection Agency* - USEPA) indica o grupo dos vírus entéricos como o indicador mais seguro e confiável para o monitoramento ambiental (Karaganis et al. 1983), uma vez que podem resistir a processos de tratamento de água e esgoto aplicados no controle bacteriano (Tree et al. 2003). O risco de infecção se agrava pela dose infectante destes agentes, que pode ser extremamente baixa, podendo variar de uma a dez unidades infecciosas (Appleton, 2000; Wyn-Jones & Sellwood, 2001; Leclerc et al. 2002). Alguns autores têm sugerido a pesquisa do HAV como um bom indicador para monitorar a qualidade virológica da água (Gersberg et al. 2006), devido à elevada resistência a tratamentos desinfectantes como a cloração (Appleton, 2000). Embora o grupo dos EV também tenha sido considerado como boa opção (Schvoerer et al. 2001), estudos recentes sugerem o uso de AdV como indicador de poluição viral humana. Os AdVs são mais estáveis no meio ambiente, podem ser evidenciados em todas as estações do ano, são de fácil detecção e não apresentam nenhuma correlação estatisticamente significativa com a quantidade dos indicadores bacterianos do grupo dos coliformes (Mehnert et al. 2001; Van Heerden et al. 2003; Jiang, 2006).

A concentração das partículas virais em amostras de água constitui uma etapa crítica para as metodologias de detecção viral, uma vez que os vírus estão presentes em pequenas quantidades na água. Em geral, são utilizadas amostras volumosas de até 1.000 litros , dependendo da origem da água a ser analisada, que devem ser reduzidas para serem testadas em volumes apropriados (Bosch, 1998; Wyn-Jones & Sellwood, 2001; Straub & Chandler, 2003).

Em geral, os procedimentos para concentração de partículas virais incluem etapas de concentração e reconcentração, onde diferentes metodologias podem ser empregadas. A concentração pode ser realizada

por adsorção-eluição das partículas virais a membranas carregadas, a matrizes de fibra de vidro ou algodão, ou ainda por floculação e ultracentrifugação. A reconcentração geralmente é obtida por floculação orgânica e inorgânica, ou por ultrafiltração (Wallis e Melnick, 1967; Payment et al. 1988; Jothikumar et al. 1993; Kittigul et al. 2001).

O uso de ensaios moleculares, particularmente da técnica de reação em cadeia pela polimerase (PCR), tem permitido novos avanços na detecção e controle de vírus entérico presentes na água. Comparada com o cultivo de células, técnica também utilizada para detecção e quantificação de vírus, a PCR apresenta várias vantagens: rapidez de execução e baixo custo, além de ser uma metodologia que apresenta alta especificidade e sensibilidade (Gilgen et al. 1997; Schvoerer et al. 2000; Gofti-Laroche et al. 2001; Frost et al. 2002). Adicionalmente, este método facilita a identificação de vírus fastidiosos, tais como RV, HAV e TTV (Abbaszadegan et al. 1999; Schvoerer et al. 2000; Wyn-Jones & Sellwwod, 2001; Carducci et al. 2003).

A análise de amostras de águas detectadas por PCR e confirmadas por *Southern-blot, nested* PCR e sequenciamento parcial do genoma, tem evidenciado, ao longo dos últimos anos, a presença destes vírus em diversos ecossistemas aquáticos (Gilgen et al. 1997; Abbaszadegan et al. 1999; Schvoerer et al. 2000; Gofti-Laroche et al. 2001; Queiroz et al. 2001; Schvoerer et al. 2001; Lee & Kim, 2002; Borchardt et al. 2003).

A principal desvantagem em relação à técnica de PCR é sua incapacidade de determinar a infecciosidade dos vírus (Bosch, 1998; Abbaszadegan et al. 1999; Schvoerer et al. 2001; Li et al. 2002). Todavia, alguns autores sugerem que a técnica de PCR detecta principalmente partículas virais intactas e não o genoma viral desprendido após a lise, pois o ácido nucléico livre tem uma estabilidade menor no ambiente, apesar de ser detectado mesmo quando os vírus são inativados por desinfecção química, calor ou proteases presentes na água (Gilgen et al. 1997; Carducci et al. 2003).

Outra desvantagem da PCR ou RT-PCR (reação em cadeia pela polimerase após transcrição reversa), voltada para detecção de vírus em água, é sua sensibilidade a inibidores da DNA polimerase tais como: proteínas, carboidratos, ácidos húmico e fúlvico e outros compostos orgânicos presentes nas amostras concentradas de água. Torna-se necessária, portanto, a remoção dos inibidores antes da detecção de vírus. Técnicas que empregam com diálise, seguida de extração com solvente e ultrafiltração, têm mostrado resultados satisfatórios na remoção de inibidores. Contudo, a PCR ainda é considerada uma boa técnica de monitoramento de contaminação viral em amostras ambientais (Abbaszadegan et al. 1999; Gofti-Laroche et al. 2001).

2. JUSTIFICATIVA

Descrito recentemente, o TTV apresenta alta prevalência e grande diversidade genética embora seu potencial patogênico ainda não tenha sido esclarecido. Desta forma, estudos sobre a caracterização molecular do TTV podem revelar genótipos, ou genogrupos, relacionados ao desenvolvimento de patogenias e fornecer dados para compreensão sobre a taxonomia do grupo.

A grande diversidade genética do TTV pode ser observada tanto em humanos quanto em animais, sendo importante a análise do genoma completo para a descrição de novas variantes. A técnica de amplificação em círculo rolante (do inglês *rolling circle amplification*, RCA) possibilita a amplificação do genoma completo de maneira independente do conhecimento prévio da seqüência-alvo. Esta técnica foi empregada com sucesso neste estudo identificando dois novos genótipos de TTV humano e um novo genogrupo de TTV suíno (artigo 1).

Devido ao caráter ubiqüitário, grande diversidade, viremia crônica, resistência e distribuição global, a presença do TTV se configura como uma fonte potencial de informação sobre a presença de contaminação gerada pela atividade humana. Neste contexto, a detecção e a concentração do TTV em amostras ambientais podem representar uma importante ferramenta para o monitoramento virológico de qualidade da água. A avaliação do papel do TTV como indicador de poluição em águas destinadas ao consumo e à recreação deve ser consolidada pela comparação da sua prevalência e concentração com a presença de outros vírus entéricos. Neste estudo, uma metodologia de concentração adequada para recuperação de um grande número de vírus foi estabelecida e aplicada no campo para avaliar a presença destes vírus. No Brasil, este constitui o primeiro trabalho descrevendo a presença de TTV no ambiente (artigo 3)..

Inicialmente, duas metodologias de concentração de partículas virais foram avaliadas utilizando o HAV como modelo (artigo 2), devido a disponibilidade de técnica molecular de quantificação previamente estabelecida. A técnica baseada na adsorção-eluição de partículas virais à

membrana de carga negativa seguida de reconcentração por ultrafiltração foi demonstrada como sendo a mais adequada para investigação de vírus em amostras de água de rio. Esta metodologia foi eleita para pesquisa destes vírus em amostras de água provenientes de igarapés urbanos na cidade de Manaus, Amazonas.

A associação desta metodologia ao protocolo de detecção pela reação em cadeia da polimerase quantitativa (qPCR) desenvolvida neste estudo permitiu a determinação da prevalência, concentração e diversidade do TTV (artigo 3) na área estudada. A utilização desta qPCR será de grande utilidade na investigação de TTV em outras matrizes ambientais, assim como em espécimes clínicos, viabilizando novas abordagens no estudo destes vírus.

O potencial do TTV como um indicador de poluição de natureza antrópica pôde ser avaliado pelo estudo comparativo da presença do TTV e de outros vírus entéricos como o HAV (artigo 4), AdV, RV, NoV e AstV (artigo 5).

3. OBJETIVOS

3.1. Objetivo Geral

Avaliar a diversidade genética e o potencial do TTV como indicador de contaminação de natureza antrópica na bacia Amazônica.

3.2. Objetivos Específicos

- Caracterizar a diversidade de genogrupos e/ou genótipos do TTV encontrados em amostras humanas e de suínos.
- Estabelecer protocolo de concentração de vírus entéricos em amostras de água utilizando o vírus da hepatite A como modelo.
- Implementar protocolo de PCR em tempo real para determinar a carga viral do TTV em amostras de água.
- Investigar a ocorrência do TTV em igarapés da bacia Amazônica utilizando o método de concentração estabelecido seguido de detecção molecular qualitativa (PCR) e quantitativa (qPCR).
- Investigar a ocorrência de outros vírus entéricos (vírus da hepatite A adenovírus, rotavírus, norovírus e astrovírus) para avaliar o potencial do TTV como marcador de poluição antrópica na área estudada.

4. RESULTADOS

4.1. ARTIGO 1

Niel C, Diniz-Mendes L, Devalle S (2005). **Rolling-circle amplification of Torque teno virus (TTV) complete genomes from human and swine sera and identification of a novel swine TTV genogroup.** *J Gen Virol*, 86:1343-1347.

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Short Communication

Rolling-circle amplification of *Torque teno virus* (TTV) complete genomes from human and swine sera and identification of a novel swine TTV genogroup

Christian Niel, Leonardo Diniz-Mendes and Sylvie Devalle

Department of Virology, Oswaldo Cruz Institute, Avenida Brasil 4365, 21040-900 Rio de Janeiro, RJ, Brazil

Correspondence
Christian Niel
niel@ioc.fiocruz.br

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Multiply primed rolling-circle amplification is a novel technology that uses bacteriophage phi29 DNA polymerase to amplify circular DNA molecules, without the need for prior knowledge of their sequences. In an attempt to detect *Torque teno virus* (TTV), rolling-circle amplification was used to amplify DNA extracted from eight human and four pig serum samples. All samples gave high molecular weight (>30 kb) amplification products. By restriction endonuclease digestion, these products generated DNA fragments whose sizes were consistent with those of human TTV (3·8 kb) and swine TTV (Sd-TTV; 2·9 kb) genomes. Two TTV isolates derived from a single AIDS patient, as well as two Sd-TTV isolates derived from a single pig, were characterized by complete nucleotide sequencing. One of the Sd-TTV isolates showed very low (43–45 %) nucleotide sequence similarity to the other Sd-TTV isolate and to the prototype isolate Sd-TTV31, and could be considered the prototype of a novel genogroup.

Torque teno virus (TTV) is a non-enveloped, single-stranded, circular DNA virus with a genomic length of 3·4–3·9 kb (Nishizawa *et al.*, 1997; Miyata *et al.*, 1999; Mushahwar *et al.*, 1999) and has been recently classified into a novel, floating genus called Anellovirus (Biagini *et al.*, 2005). TTV is found in the plasma of >80 % of the human population worldwide (Prescott & Simmonds, 1998; Takahashi *et al.*, 1998; Niel *et al.*, 1999). Co-infection of single individuals with multiple TTV isolates is frequent (Takayama *et al.*, 1999; Niel *et al.*, 2000). TTV has a wide genetic diversity and virus isolates have been classified into five main phylogenetic groups (1–5) with low nucleotide sequence similarity between them (Peng *et al.*, 2002). Anelloviruses are not restricted to human hosts and have also been detected in non-human primates, tupaias, cats, dogs and pigs (Leary *et al.*, 1999; Verschoor *et al.*, 1999; Okamoto *et al.*, 2001, 2002). However, few complete nucleotide sequences from animal TTVs have been reported.

In their natural replication cycle, some DNA viruses, like circoviruses, employ a rolling-circle mechanism to propagate their circular genomes. Multiply primed rolling-circle amplification is a novel technique able to amplify circular DNA molecules such as plasmids with great efficiency (Dean *et al.*, 2001). The method utilizes bacteriophage phi29 DNA polymerase, a high-fidelity enzyme, with a strong strand-displacing capability, high processivity and proofreading activity (Garmendia *et al.*, 1992; Esteban *et al.*, 1993). Unlike PCR, the primers used in the amplification reaction are random hexamers. Previous knowledge of the nucleotide sequences to be amplified therefore is not necessary. Furthermore, phi29 DNA polymerase is very stable, with linear kinetics at 30 °C for over 12 h, eliminating the need for thermal cycling. The reaction products are high molecular weight, linear, double-stranded, tandem-repeat copies of the input DNA that can subsequently be digested with restriction endonucleases.

In this study, multiply primed rolling-circle amplification was used to amplify the complete genomes of human TTV and porcine (*Sus domesticus*) TTV (Sd-TTV) after direct extraction of viral DNA from serum. A novel genogroup of Sd-TTV was identified.

To evaluate the utility of the rolling-circle amplification technique for identification of TTV, eight human serum samples were used, originating from four voluntary blood donors and four patients with AIDS. All the subjects lived in Rio de Janeiro, Brazil, and were 25–35 years old. In addition, serum samples collected from four adult, randomly selected pigs belonging to a single herd from the state of Rio de Janeiro were used.

Viral DNA was extracted from 250 µl serum as described previously (Niel *et al.*, 1994) and resuspended in 30 µl distilled water. For multiply primed rolling-circle

The GenBank/EMBL/DDBJ accession numbers of the sequences reported in this paper are AY823988–AY823991.

amplification, 5 µl DNA was denatured for 3 min at 95 °C, cooled to 30 °C and added to a mixture containing 5 U phi29 DNA polymerase (New England Biolabs), 50 µM random primers (Invitrogen), 1 mM each dNTP (Amersham Biosciences) and 200 µg BSA ml^{-1} in a final volume of 100 µl of reaction buffer [50 mM Tris/HCl, pH 7·5, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM dithiothreitol]. Amplification was for 18 h at 30 °C, followed by 10 min at 65 °C to inactivate the phi29 DNA polymerase.

Ten microlitres of amplification product was digested with 10 U *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I or *Pvu*II. Restriction digests were separated by 1 % agarose gel electrophoresis and visualized by UV light exposure after ethidium bromide staining.

*Eco*RI fragments showing sizes corresponding to those of TTV (~4 kb) and Sd-TTV (3 kb) were purified using the QIAquick gel extraction kit (Qiagen). Fifty nanograms of purified DNA was ligated to 5 ng plasmid vector pUC19 linearized with *Eco*RI. After transformation of TOP10 *Escherichia coli* (Invitrogen), the bacteria were incubated for blue/white colony screening on agar plates containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and ampicillin.

Bacteria carrying recombinant plasmids were grown overnight. Plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen). Inserts were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequencing primers were universal and reverse M13 primers, as well as primers designed specifically to be used for genome walking.

Firstly, the minimal quantity of enzyme required to achieve successful multiply primed rolling-circle amplification assays was determined. Using 1 pg of plasmid pUC19 as input DNA, 1 U of enzyme (in a 20 µl reaction volume) was sufficient to generate a strong DNA band of high molecular mass (>30 kb). Secondly, the sensitivity of the method was determined with decreasing amounts of pUC19. A faint DNA band was visible on the gel using as little as 1 fg of plasmid (about 350 copies) in the amplification mixture (not shown).

To determine whether the rolling-circle amplification method could be used for detection of TTV in human serum, assays were performed with DNA extracted from serum samples collected from four blood donors and four AIDS patients. High molecular mass DNA was obtained in all cases. DNA samples were digested with five restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I or *Pvu*II), chosen from those having a 6 bp recognition site and, consequently, a high probability of having a unique restriction site in a 3·8 kb DNA molecule (the size of the TTV genome). The results are shown in Fig. 1. In all

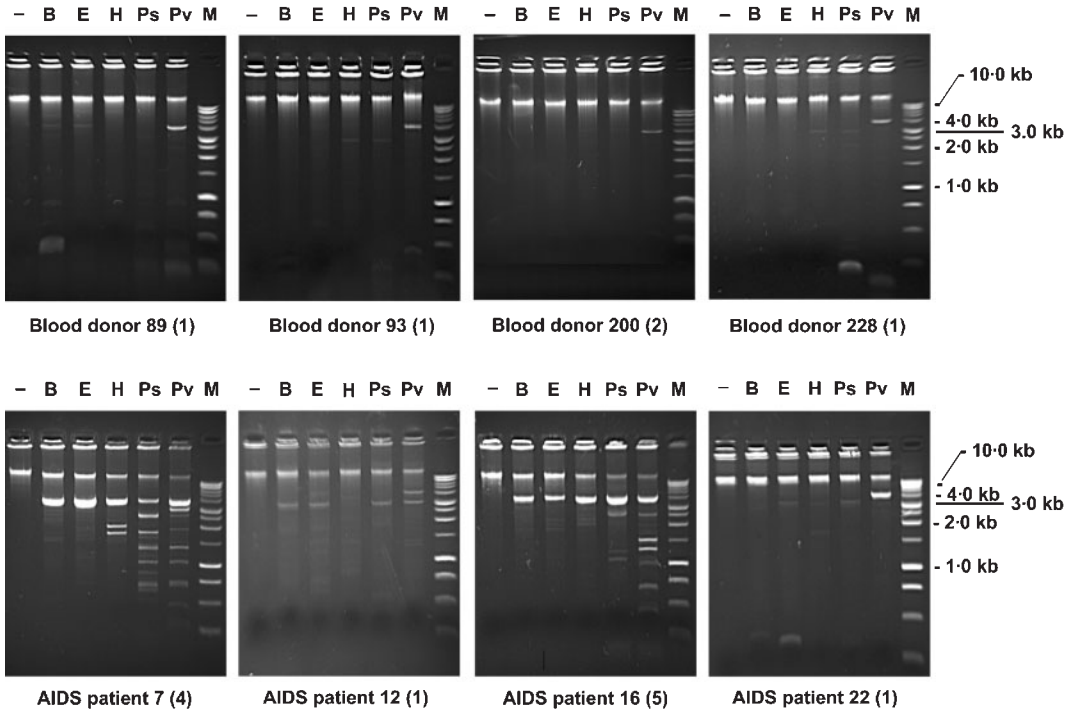

Fig. 1. Agarose gel (1 %) electrophoresis of rolling-circle amplification products derived from sera collected from blood donors and patients with AIDS. Numbers in parentheses refer to the numbers of different TTV genogroups previously detected by PCR (Devalle & Niel, 2004). Lanes show undigested amplification product (–) and amplification product digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Pst*I (Ps) or *Pvu*II (Pv). M, 1 kb DNA molecular mass marker (Promega).

samples, a band of 4 kb (or slightly shorter) was seen after digestion of rolling-circle amplification products with at least one restriction endonuclease. The eight samples under study had been previously tested by PCR for the presence of TTV isolates belonging to each of the five phylogenetic groups (Devalle & Niel, 2004). At that time, it was noticed that AIDS patients 7 and 16 were co-infected with TTV isolates belonging to four and five genogroups, respectively, whereas TTV from only one genogroup was detected in blood donors 89, 93 and 228, as well as in AIDS patients 12 and 22 (two genogroups were identified in blood donor 200). It was therefore remarkable that the rolling-circle amplification products derived from patients 7 and 16 generated a number of restriction fragments notably higher than observed in the six other individuals (Fig. 1).

In the same way, four swine serum samples were submitted to viral DNA extraction, rolling-circle amplification and restriction endonuclease digestion. For all samples, restriction fragments of approximately 3 kb, consistent with the size of the Sd-TTV genome, were observed after separate digestions with at least three restriction endonucleases (Fig. 2).

*Eco*RI fragments of approximately 4 and 3 kb in size, derived from AIDS patient 7 and pig 845, respectively, were cloned into the plasmid vector pUC19. Two recombinant human clones (2h and 3h) and two recombinant pig clones (1p and 2p) were selected and their nucleotide sequences determined. All four clones showed a genetic organization characteristic of TTV, with (i) a coding region covering about two-thirds of the genome and containing four (human clones) or three (swine clones) open reading frames; (ii) a GC-rich stretch in the untranslated region (UTR); and (iii) a TATA box, located upstream of the smallest open reading frame (not shown).

Human clones 2h and 3h had genomic lengths of 3816 and 3920 nt, respectively. They shared >60 % sequence identity with each other and with human TTV isolate TYM9 belonging to genogroup 3, but <60 % with isolates representative of the other four genogroups. Clones 2h and 3h were thus classifiable into genogroup 3.

Clones 1p and 2p, although derived from a single pig, shared a very low (43·4 %) sequence identity. To date, one swine TTV isolate (Sd-TTV31), from Japan, has been fully characterized at the genome level and sequences with a length of 69–80 nt, localized in the UTR, have been determined for eight isolates (Okamoto *et al.*, 2002). Clone 1p had a genomic length of 2872 nt, very similar to Sd-TTV31 (2878 nt) and shared a relatively high similarity (69·6 %) with that prototype isolate. However, isolate 2p (2735 nt) showed very low (45·1 %) sequence identity with Sd-TTV31. Actually, clone 2p was almost as distantly related to Sd-TTV31 as it was to TTVs infecting tupaias, cats, dogs and humans (Table 1). Nevertheless, within the small genome segment (69–80 nt) mentioned above, the sequence of clone 2p was identical to those of Japanese isolates Sd-TTV83 and Sd-TTV161 (not shown).

Recent studies have described the multiply primed rolling-circle amplification of complete begomovirus (Inoue-Nagata *et al.*, 2004) and papillomavirus (Rector *et al.*, 2004) genomes. In both cases, amplification was performed using a commercial DNA amplification kit. The amplification assays described here were performed with recombinant phi29 DNA polymerase, not included in a commercial kit. The method was inexpensive and sensitive. As little as 1 fg (about 350 copies) of input plasmid pUC19 was sufficient to produce a visible band (>5 ng of DNA) in an agarose gel.

This study is the first to report the rolling-circle amplification of a complete viral genome from a biological fluid. All serum samples tested gave a high molecular weight (>30 kb) DNA band, revealing the high efficiency of the method for amplification of viral DNA present in serum. Restriction fragments of approximately 4 kb (the size of the TTV genome) were found in all eight human samples tested (Fig. 1). Whether other viral genomes were amplified in our experiments is not known, for at least two reasons. Firstly,

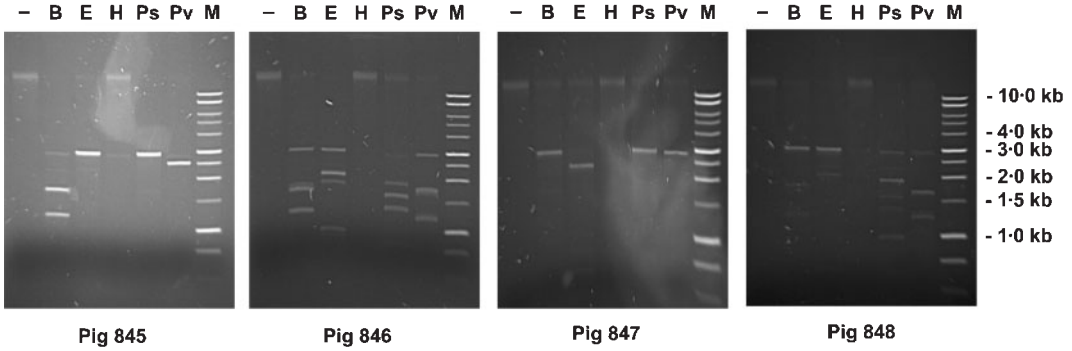

Fig. 2. Agarose gel (1 %) electrophoresis of rolling-circle amplification products derived from swine sera. Lanes show undigested amplification product (–) and amplification product digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Pst*I (Ps) or *Pvu*II (Pv). M, 1 kb DNA molecular mass marker (Promega).

Table 1. Pairwise percentage nucleotide identity comparisons of full-length nucleotide sequences of TTVs infecting different mammalian species

Percentage identity >60 % is shown in bold.

Isolate	Identity (%) with:						
	Tbc-TTV14 (tupaia)	Fc-TTV4 (cat)	Cf-TTV10 (dog)	TA278 (human)	Sd-TTV31 (swine)	Clone 1p (swine)	Clone 2p (swine)
Tbc-TTV14	100·0	38·3	41·6	39·5	43·6	43·1	41·6
Fc-TTV4	–	100·0	38·2	39·4	39·1	38·5	36·8
Cf-TTV10	–	–	100·0	38·8	38·8	38·9	42·5
TA278	–	–	–	100·0	40·4	41·4	42·6
Sd-TTV31	–	–	–	–	100·0	**69·6**	45·1
Clone 1p	–	–	–	–	–	100·0	43·4
Clone 2p	–	–	–	–	–	–	100·0

the restriction fragments whose size did not correspond to the TTV genome were not analysed. Some may have resulted from digestion of TTV DNA molecules into two or more fragments, while others may have had other origins. Secondly, unlike PCR, multiply primed rolling-circle amplification is a generic method that does not favour certain DNA molecules with regard to sequence. Circular genomes of blood-borne viruses other than TTV may therefore have been amplified, although not visualized on the agarose gel, due to the low amounts in the samples tested.

A significant difference was observed between blood donors and AIDS patients, with a higher number of restriction fragments observed in the case of AIDS patients. This was particularly true for patients 7 and 16, who were co-infected with TTV isolates from different genogroups (Devalle & Niel, 2004). Further studies are necessary to confirm this correlation between co-infection and complexity of the restriction patterns of amplification products. A high TTV load in human immunodeficiency virus-positive samples (Christensen *et al.*, 2000; Shibayama *et al.*, 2001) could also facilitate TTV detection by rolling-circle amplification.

Due to the singularly high genetic diversity of human TTV, no truly 'universal' PCR system, able to detect all isolates, has been developed. Multiply primed rolling-circle amplification, as a sequence-independent strategy for detection of circular DNA viruses, should be useful for identification of novel genogroups.

Recombination between different TTV isolates co-infecting single individuals has been proposed, based on phylogenetic analysis of nucleotide sequences available from databases (Worobey, 2000; Manni *et al.*, 2002). However, this has been questioned, due to possible artefacts (Jelcic *et al.*, 2004). Indeed, all TTV nucleotide sequences deposited in databases have been obtained through sequencing of PCR products covering part, not the whole, of the genomes. In this way, a number of 'complete sequences' available from the databases may result from combinations of partial sequences obtained from different DNA molecules. Nucleotide sequencing of complete TTV genomes obtained through rolling-circle amplification assays circumvents this problem.

Sd-TTV was first recognized in Japan (Okamoto *et al.*, 2002) and, more recently, in North America, Spain, China, Korea and Thailand (McKeown *et al.*, 2004). The present work, showing that 4/4 pigs from a single Brazilian herd were Sd-TTV-infected, confirms the global distribution of the virus. The large genetic distance existing between clone 2p on the one hand, and clone 1p and prototype strain Sd-TTV31 on the other, shows that swine TTV has a wide genetic diversity, like its human counterpart. In accordance with the criteria used to classify human TTV isolates, clone 2p (Sd-TTV2p) could be considered the prototype of a novel genogroup. Sd-TTV31 and Sd-TTV2p would thus be the prototypes of Sd-TTV genogroups 1 and 2, respectively.

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References

Biagini, P., Todd, D., Bendinelli, M. & 8 other authors (2005). Anellovirus. In *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses*, pp. 335–341. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball. London: Academic Press.

Christensen, J. K., Eugen-Olsen, J., Sorensen, M., Ullum, H., Gjedde, S. B., Pedersen, B. K., Nielsen, J. O. & Krogsgaard, K. (2000). Prevalence and prognostic significance of infection with TT virus in patients infected with human immunodeficiency virus. *J Infect Dis* **181**, 1796–1799.

Dean, F. B., Nelson, J. R., Giesler, T. L. & Lasken, R. S. (2001). Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. *Genome Res* **11**, 1095–1099.

Devalle, S. & Niel, C. (2004). Distribution of TT virus genomic groups 1–5 in Brazilian blood donors, HBV carriers, and HIV-1-infected patients. *J Med Virol* **72**, 166–173.

Esteban, J. A., Salas, M. & Blanco, L. (1993). Fidelity of phi 29 DNA polymerase. Comparison between protein-primed initiation and DNA polymerization. *J Biol Chem* **268**, 2719–2726.

Garmendia, C., Bernad, A., Esteban, J. A., Blanco, L. & Salas, M. (1992). The bacteriophage phi 29 DNA polymerase, a proofreading enzyme. *J Biol Chem* **267**, 2594–2599.

Inoue-Nagata, A. K., Albuquerque, L. C., Rocha, W. B. & Nagata, T. (2004). A simple method for cloning the complete begomovirus genome using the bacteriophage ϕ29 DNA polymerase. *J Virol Methods* **116**, 209–211.

Jelcic, I., Hotz-Wagenblatt, A., Hunziker, A., zur Hausen, H. & de Villiers, E. M. (2004). Isolation of multiple TT virus genotypes from spleen biopsy tissue from a Hodgkin's disease patient: genome reorganization and diversity in the hypervariable region. *J Virol* **78**, 7498–7507.

Leary, T. P., Erker, J. C., Chalmers, M. L., Desai, S. M. & Mushahwar, I. K. (1999). Improved detection systems for TT virus reveal high prevalence in humans, non-human primates and farm animals. *J Gen Virol* **80**, 2115–2120.

Manni, F., Rotola, A., Caselli, E., Bertorelle, G. & Luca, D. D. (2002). Detecting recombination in TT virus: a phylogenetic approach. *J Mol Evol* **55**, 563–572.

McKeown, N. E., Fenaux, M., Halbur, P. G. & Meng, X. J. (2004). Molecular characterization of porcine TT virus, an orphan virus, in pigs from six different countries. *Vet Microbiol* **104**, 113–117.

Miyata, H., Tsunoda, H., Kazi, A., Yamada, A., Khan, M. A., Murakami, J., Kamahora, T., Shiraki, K. & Hino, S. (1999). Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. *J Virol* **73**, 3582–3586.

Mushahwar, I. K., Erker, J. C., Muerhoff, A. S., Leary, T. P., Simons, J. N., Birkenmeyer, L. G., Chalmers, M. L., Pilot-Matias, T. J. & Dexai, S. M. (1999). Molecular and biophysical characterization of TT virus: evidence for a new virus family infecting humans. *Proc Natl Acad Sci U S A* **96**, 3177–3182.

Niel, C., Moraes, M. T., Gaspar, A. M., Yoshida, C. F. & Gomes, S. A. (1994). Genetic diversity of hepatitis B virus strains isolated in Rio de Janeiro, Brazil. *J Med Virol* **44**, 180–186.

Niel, C., de Oliveira, J. M., Ross, R. S., Gomes, S. A., Roggendorf, M. & Viazov, S. (1999). High prevalence of TT virus infection in Brazilian blood donors. *J Med Virol* **57**, 259–263.

Niel, C., Saback, F. L. & Lampe, E. (2000). Coinfection with multiple TT virus strains belonging to different genotypes is a common event in healthy Brazilian adults. *J Clin Microbiol* **38**, 1926–1930.

Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y. & Mayumi, M. (1997). A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochem Biophys Res Commun* **241**, 92–97.

Okamoto, H., Nishizawa, T., Tawara, A., Peng, Y., Kishimoto, J. & Wang, Y. (2001). Genomic and evolutionary characterization of TT virus (TTV) in tupaias and comparison with species-specific TTVs in humans and non-human primates. *J Gen Virol* **82**, 2041–2050.

Okamoto, H., Takahashi, M., Nishizawa, T., Tawara, A., Fukai, K., Muramatsu, U., Naito, Y. & Yoshikawa, A. (2002). Genomic characterization of TT viruses (TTVs) in pigs, cats and dogs and their relatedness with species-specific TTVs in primates and tupaias. *J Gen Virol* **83**, 1291–1297.

Peng, Y. H., Nishizawa, T., Takahashi, M., Ishikawa, T., Yoshikawa, A. & Okamoto, H. (2002). Analysis of the entire genomes of thirteen TT virus variants classifiable into the fourth and fifth genetic groups, isolated from viremic infants. *Arch Virol* **147**, 21–41.

Prescott, L. E. & Simmonds, P. (1998). Global distribution of transfusion-transmitted virus. *N Engl J Med* **339**, 776–777.

Rector, A., Tachezy, R. & van Ranst, M. (2004). A sequence-independent strategy for detection and cloning of circular DNA virus genomes by using multiply primed rolling-circle amplification. *J Virol* **78**, 4993–4998.

Shibayama, T., Masuda, G., Ajisawa, A., Takahashi, M., Nishizawa, T., Tsuda, F. & Okamoto, H. (2001). Inverse relationship between the titre of TT virus DNA and the CD4 cell count in patients infected with HIV. *AIDS* **15**, 563–570.

Takahashi, K., Hoshino, H., Ohta, Y., Yoshida, N. & Mishiro, S. (1998). Very high prevalence of TT virus (TTV) infection in general population of Japan revealed by a new set of PCR primers. *Hepatol Res* **12**, 233–239.

Takayama, S., Yamazaki, S., Matsuo, S. & Sugii, S. (1999). Multiple infection of TT virus (TTV) with different genotypes in Japanese hemophiliacs. *Biochem Biophys Res Commun* **256**, 208–211.

Verschoor, E. J., Langenhuijzen, S. & Heeney, J. L. (1999). TT viruses (TTV) of non-human primates and their relationship to the human TTV genotypes. *J Gen Virol* **80**, 2491–2499.

Worobey, M. (2000). Extensive homologous recombination among widely divergent TT viruses. *J Virol* **74**, 7666–7670.

4.2. ARTIGO 2

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Evaluation of methods used to concentrate and detect hepatitis A virus in water samples

Livia Melo Villar*, Vanessa Salete de Paula, Leonardo Diniz-Mendes, Elisabeth Lampe, Ana Maria Coimbra Gaspar

Department of Virology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Av. Brazil 4365, 21045-900 Rio de Janeiro, RJ, Brazil

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Abstract

Two adsorption–elution concentration methods, both involving negatively charged membranes, were evaluated in order to monitor hepatitis A virus (HAV) contamination in tap, river, mineral and coastal water samples: elution with urea–arginine phosphate buffer/reconcentration with magnesium chloride (method 1); and sodium hydroxide elution/reconcentration with a commercial concentrator (method 2). Nested (qualitative) reverse transcriptase PCR (RT-PCR) and real-time (quantitative) RT-PCR were used to detect and quantify HAV RNA in concentrated water samples. For concentrating HAV, method 1 was found to be the most suitable for tap water and method 2 most suitable for mineral water. HAV inoculated experimentally was detected in river water samples by both methods and in coastal water samples by neither method. The detection limits were 6×10^9 g equiv./ml for qualitative PCR and 60 g equiv./ml for quantitative PCR. In a field application study, HAV was detected in 20% of river and tap water samples but not in coastal or mineral water samples. River water samples contained subgenotype IA, and tap water samples contained subgenotype IB. It is concluded that, although influencing qualitative PCR, the concentration method does not affect quantitative PCR, which could therefore be used for all types of water samples. Both techniques are recommended for detecting HAV in environmental water samples.

Keywords: Hepatitis A virus; Water; Concentration; Quantitative RT-PCR

1. Introduction

Hepatitis A virus (HAV) is an enterically transmitted pathogen that is a public health concern worldwide, causing substantial morbidity, principally in developing countries (Tanaka, 2000). The HAV genome is a positive-strand RNA of approximately 7.5 kb with a single open reading frame encoding a large polyprotein containing approximately 2230 amino acids (Koff, 1998). The HAV strains isolated from various parts of the world constitute a single serotype and are divided into six genotypes based on phylogenetic analysis of nucleotide sequences in the VP1/2A region (Robertson et al., 1992; Lu et al., 2004). Most of the human strains studied belong to genotypes I or III. The majority (80%) belong to genotype I (Robertson et al., 1992).

Since HAV is excreted in large quantities in feces, it has also been found in sewage water (Vaidya et al., 2002; Morace et al., 2002), river water (Taylor et al., 2001) and ocean water (Brooks et al., 2005). The presence of HAV in these waters is a public health concern because, even at low concentrations, HAV can cause illness (Cuthbert, 2001). Generally, microbiological water quality is assessed using bacterial indicators such as heterotrophic bacteria and coliforms. However, Brooks et al. (2005) found no relationship between HAV concentration and fecal bacterial coliform contamination.

Most studies on HAV involving acute cases of hepatitis are based on epidemiological evaluation and virus detection in clinical samples from patients (Normann et al., 1995; Robertson et al., 2000; Villar et al., 2002, 2004a; Poovorawan et al., 2005). Due to lack of sensitive methods, water is not tested routinely for the presence of viruses. Combining information on the level of HAV contamination in the environment with epidemiological data would make it easier to identify the source of contamination during an outbreak.

Among the various methods suggested for concentrating waterborne viruses, adsorption–elution using micropore filters seems to be the most accurate (Kittigul et al., 2001, 2005). The

* Corresponding author. Tel.: +55 21 2598 4483; fax: +55 21 2270 6397.
E-mail address: lvillar@ioc.fiocruz.br (L.M. Villar).

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PCR method had been used to detect HAV in environmental samples and is one of the most sensitive methods available for viral monitoring (Abbaszadegan et al., 1999; Katayama et al., 2002; Vaidya et al., 2002).

In this study, two adsorption–elution methods, both using negatively charged membranes, were evaluated as to their ability to concentrate HAV in water samples from four different sources (coastal water, river water, tap water and mineral water). In addition, applicability of nested (qualitative) RT-PCR and real-time (quantitative) RT-PCR in detecting and enumerating HAV RNA were evaluated for environmental samples in which HAV was concentrated using these methods.

2. Materials and methods

2.1. Virus stocks

The HAV strain HAF-203 was propagated in FRhK-4 cells (Gaspar et al., 1993). Cultures were stored in plastic bottles for 7 days at 37 °C in an atmosphere of 5% CO_2. Cell-associated virus was extracted by freeze-thawing the bottle contents, including the medium supernatant (William's medium E supplemented with 2% heat-inactivated fetal bovine serum obtained from Sigma Chemical Co., St. Louis, MO, USA). This procedure was performed in triplicate. The harvested fluid was subjected to a short burst of sonication followed by a brief low-speed centrifugation. The supernatant was aliquoted and stored at −70 °C. The concentration of virus was determined by in situ enzyme immunoassay (Villar et al., 2004b), and virus presenting a titer of 5.024 log $TCID_{50}$/ml was used as the stock solution for the evaluation of the concentration methods.

2.2. Water samples

Each sample consisted of 2.5 l of water collected in sterile bottles to standardize the methods. River water was collected from the Tijuca National Park (in the city of Rio de Janeiro), coastal water was collected from the São Conrado coast, tap water was collected from a residence located in the metropolitan region of the city of Rio de Janeiro, and a single brand of mineral water was evaluated. All samples were collected in the daytime and stored in ice baths until testing. Tap water was dechlorinated with sodium thiosulfate (Sigma Chemical Co.) at a final concentration of 50 mg/l. All samples presented acceptable levels of total and fecal coliforms.

Both HAV concentration methods were used to test their applicability for the testing of field samples. A 2.5 l samples of each of the following were collected: water from a polluted river (the Faria Timbó) located in the metropolitan region of the city of Rio de Janeiro (five samples); tap water from a school that experienced an outbreak of hepatitis A in 2004 (five samples); mineral water (five samples of a single brand); and coastal water (five samples). Water samples were collected in the daytime and immediately transported to the laboratory for virus concentration.

2.3. Concentration methods

Each water sample was inoculated with 1 or 5 or 10 μl of HAV (strain HAF-203) to estimate the virus recovery using the two concentration methods. Both methods, designated methods 1 and 2, were carried out as described previously by Jothikumar et al. (1993) and Katayama et al. (2002).

Method 1 was modified by adding $MgCl_2{\cdot}6H_2O$ (at a final concentration of 1200 mg/l in 500 ml of the water sample) and by adjusting the pH to 5.0. The samples were filtered through 125 mm filters with a pore size of 0.45 μm (Stericup; Millipore, Bedford, MA, USA). A 10 min soak in urea–arginine phosphate buffer, pH 9.0, was used for viral elution. The eluates were then run through the filter. Flocculation was achieved by adding 200 μl 1 M $MgCl_2{\cdot}6H_2O$. The samples were centrifuged for 30 min at 3000 × *g*, and the pellets were solubilized in 800 μl of distilled sterile water.

Method 2 involved the use of a type HA negatively charged membrane (Millipore), with a 0.45 μm pore size and a 142 mm diameter, and a vacuum pump system. In this method, $MgCl_2{\cdot}6H_2O$ was added (in a final concentration of 1200 mg/l in 2 l of the water sample) and the pH was adjusted to 5.0. Two liters of water sample were filtered to adsorb the viruses to the membrane, and the cation was extracted by passing 300 ml of 0.5 mM H_2SO_4 through the membrane. Subsequently, 10 ml of 1 mM NaOH (pH 10.5–10.8) was poured over the membrane, and the filtrate was recovered in a tube containing 0.1 ml of 50 mM H_2SO_4 and 0.1 ml of 100× TE buffer for neutralization. The samples were stored at −20 °C until further processing.

All 10 ml of the eluate were introduced into a Centriprep 50 Concentrator (Millipore) and filtered at 1500 × *g* for 10 min at 4 °C. After the filtrate had been removed, the concentrate was rinsed twice with 10 ml of Milli-Q water using the same procedure, and a final volume of 2 ml was obtained.

$MgCl_2$ was used to adsorb the virus with both methods and for all samples. The tap water was dechlorinated with sodium thiosulfate in a final concentration of 50 mg/l. All samples were tested in triplicate.

2.4. Preventing cross-contamination of viruses during the concentration procedure

Particular care was taken in order to prevent samples from contamination with virus from extraneous sources and to prevent cross-contamination of samples during filtration. Precautions included a decontamination procedure, using the vacuum pump system to recirculate 0.3 mM of free chlorine solution for 15 min, followed by dechlorination with 0.3 mM of sodium thiosulphate solution for 3 min.

2.5. Qualitative nested reverse transcription PCR and sequencing

Viral RNA was extracted from 140 μl of the eluate using the Qiagen Viral RNA KIT (Qiagen, Valencia, Spain). Reverse tran-

scription was carried out in the presence of 20 pmol of random primer (Invitrogen, Rockville, MD, USA) and 10 μl of RNA using 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a final volume of 20 μl at 37 °C for 1 h, followed by a 10 min incubation at 65 °C. Half of the cDNA was used in nested RT-PCR for the VP1/2A region with degenerated primers as described elsewhere (De Paula et al., 2002). The PCR products (10 μl) were loaded onto a 2% agarose gel, electrophoresed and stained with ethidium bromide to visualize bands (expected length, 247 bp). Amplicons were purified using the QIAquick Gel extraction kit (Qiagen) according to the manufacturer recommendations. The HAV RNA-positive field samples were further sequenced. Direct nucleotide sequencing reaction was performed in both directions with a Big Dye Terminator kit (Applied Biosystems, Foster City, CA, USA) and an automatic DNA sequencer (ABI Prism 310; Applied Biosystems). The sequences reported in this paper have been deposited in the GenBank sequence database under the accession numbers DQ251459 and DQ251460.

2.6. Sequence analysis

Algorithms within the GCG software package, version 10.1 (Wisconsin Sequence Analysis Package; Genetic Computer Group, Madison, WI, USA), were used for editing, alignment of nucleotides and translation of nucleotides into amino acid sequences. To construct a phylogenetic tree, sequences from 218 VP1/2A junction nucleotides (positions 3024–3191) were aligned using the CLUSTAL X program (Thompson et al., 1997). A matrix was then generated to construct the Kimura two-parameter model (Felsenstein, 1993). Using this matrix and the neighbor-joining method (Saitou and Nei, 1987), a phylogenetic tree was generated, the reliability of which was assessed by bootstrap resampling (1000 pseudoreplicates). These methods were implemented using the MEGA 2.1 program (Kumar et al., 2001).

2.7. Preventing PCR contamination

PCR amplification was carried out in a separate room to avoid carryover PCR contamination. Each PCR experiment included distilled water as a negative control. No amplification was observed in the negative controls, indicating the absence of aerosol or reagent contamination. None of the negative controls in the experiments resulted in false-positive results, and no spurious bands representing nonspecific amplification were observed. The post-PCR detection procedures, such as electrophoresis, were also carried out in a separate room away from where the PCR reactions were carried out, thereby preventing PCR contamination.

2.8. Quantitative PCR standard curve

The HAF-203 strain was extracted and amplified using 5′ noncoding region primers, forward primer numbering as wild-type HM-175 (accession number, M14707) positions 112–135 5′-TTTTCCCTTTCCTATTCCCTTTGT-3′ and reverse positions 488–511 5′-AGTCCTCCGGCGTTGAATGGTTTT-3′. The 400 bp fragment was sequenced to identify HAV RNA. The amplicon was then cloned into a TOPO cloning vector (Invitrogen). This plasmid DNA, which is derived from a recombinant clone, was diluted and used as standard curve points (a positive control) for HAV in the real-time RT-PCR.

2.9. Assay by design

The 5′ noncoding region of the HAV genome was chosen to design the TaqMan assay. The primers and probe were identified using the Primer Express program (TaqMan gene expression assay), forward primer numbering as wild-type HM-175 (accession number M14707) positions 157–180 5′-CTGCAGGTTCAGGGTTCTTAAATC-3′, reverse primers positions 219–240 5′-GAGAGCCCTGGAAGAAAGAAGA-3′ and HAV-probes 198–218 (FAM-5′-ACTCATTTTTCACGCTTTCTG-3′).

All PCR reactions were carried out using the 7500 real-time PCR system (Applied Biosystems). The RT step for the real-time assay was performed as described above for qualitative PCR. For each PCR run, a master mix was prepared on ice with 1× TaqMan Universal PCR Master Mix (Roche, Nutley, NJ, USA) and 1.25 ml of assay (300 nM each primer, 150 nM probe) (Gentec, Applied Biosystems Assay, Foster City, CA, USA). Five microliters of cDNA from water samples and 1 μl of standard curve points were added to 20 μl of the PCR master mix. The thermal cycling conditions comprised an initial step at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min.

2.10. Quality standards of qualitative PCR

A 10-fold dilution of the HAV strain HAF-203 in ultrapure water was extracted from 140 μl of each sample to determine the sensitivity of the technique in the same way as described previously. Five non-HAV samples were evaluated, including RNA from two hepatitis E virus isolates and DNA from three torque teno virus isolates to test the specificity of HAV primers. The ability of the assay to detect HAV strains other than HAF-203 (subgenotype IB) was confirmed by a positive response with a clinical isolate from subgenotype IA (RJ-12). HAV was added to concentrated negative samples, which were then submitted to nested PCR to evaluate the potential for inhibition of the RT-PCR.

2.11. Quality standards for quantitative PCR

Experiments were conducted to determine the sensitivity and specificity of the assay. The sensitivity of the real-time RT-PCR assay in detecting HAV RNA was investigated by using serial dilutions of the HAV strain HAF-203 in ultrapure water (Invitrogen). The specificity of this assay and its ability to detect other HAV strains were evaluated in the same way as described for nested RT-PCR.

3. Results

3.1. Concentration of HAV RNA in artificially spiked water samples

The sensitivity of the concentration method was tested by spiking each 2.5 l sample (of tap, river, coastal and mineral water) with 1 μl (6×10^6 genomes), 5 μl (3×10^7 genomes) or 10 μl (6×10^7 genomes) of the HAV strain HAF-203. Using qualitative PCR and method 1, we detected 6×10^6 genomes in tap water and 6×10^7 genomes in mineral water. Using qualitative PCR and method 2, we detected 6×10^7 genomes in tap water and 6×10^6 genomes in mineral water. In river water samples, we detected 6×10^6 genomes regardless of the concentration method used. However, inoculated HAV was not detected in coastal samples using either method (Table 1). In addition, no amplicon was detected in filtrate samples, showing that there was no significant loss of HAV RNA.

Quantitative PCR was carried out for the detection and quantification of HAV in water samples. Using method 1, HAV was detected in all water sample types with the exception of two coastal samples inoculated with 1 and 5 μl of HAV (Table 1). Using method 2, HAV was detected successfully in all water sample types except for one coastal water sample inoculated with 1 μl of HAV (Table 1). Therefore, all but one of the samples that tested positive in the qualitative PCR also was successfully amplified by quantitative PCR. In addition, seven concentrated samples that tested negative in the qualitative PCR tested positive in the quantitative PCR (Table 1).

3.2. Sensitivity and specificity of qualitative PCR

In the 10-fold serial dilutions of HAF-203 in distilled water, the amplification reactions produced the expected 247 bp fragments. The detection limit for this method was 10^{-9}-fold dilution (Fig. 1).

The specificity of the assay was evaluated by testing five non-HAV samples and no false-positive amplification was observed. In addition, the HAV strain RJ-12 was detected efficiently using this technique (data not shown). These results confirmed the specificity of these primers, which have been tested extensively and found to be highly specific for hepatitis A virus (De Paula et al., 2002, 2004b; Villar et al., 2004a).

The HAV strain HAF-203 was added at the limit of the technique in concentrated water samples that did not present any amplification to evaluate PCR inhibitors in environmental water samples and the samples were then amplified using nested PCR. After amplification, positive results were obtained in all water

Table 1
Detection of HAV RNA in four types of water using two methods of concentration (Jothikumar et al., 1993, method 1 and Katayama et al., 2002, method 2) and two methods of detection (real-time RT-PCR and nested RT-PCR)

Spiked HAV[a]	Real-time RT-PCR (genome/ml)			Nested RT-PCR		
	Initial HAV concentration in artificial water (2500 ml)[b]	Method 1 final HAV concentration in artificial water (2 ml)[b]	Method 2 final HAV concentration in artificial water (2 ml)[b]	Initial HAV concentration in artificial water (2500 ml)[b]	Method 1 final HAV concentration in artificial water (2 ml)[b]	Method 2 final HAV concentration in artificial water (2 ml)[b]
Tap						
1 μl	1740	3900	659	−	+	−
5 μl	2230	4380	1570	−	+	−
10 μl	2910	5265	3930	+	+	+
Mean ± S.D.	2293 ± 587.6	4515 ± 692.4	2053 ± 1688.1	ND	ND	ND
Mineral						
1 μl	1714	1915	2310	−	−	+
5 μl	2075	2205	2615	−	−	+
10 μl	2280	2900	7210	+	+	+
Mean ± S.D.	2023 ± 286.5	2340 ± 506.19	4045 ± 2745.2	ND	ND	ND
River						
1 μl	972	2405	2520	−	+	+
5 μl	2170	2620	2725	−	+	+
10 μl	2565	7305	3205	+	+	+
Mean ± S.D.	1902.3 ± 829.5	4110 ± 2769.0	2816.6 ± 351.5	ND	ND	ND
Coastal						
1 μl	1151	Undetermined	Undetermined	−	−	−
5 μl	1225	Undetermined	71.7	−	−	−
10 μl	2960	865	75	−	−	−
Mean ± S.D.	1778.6 ± 1023.7	ND	ND	ND	ND	ND

SD: standard deviation; ND: not done
[a] HAV inoculated in 2.5 l of water sample/1 μl = 6×10^6 genomes.
[b] Tests were run in triplicate.

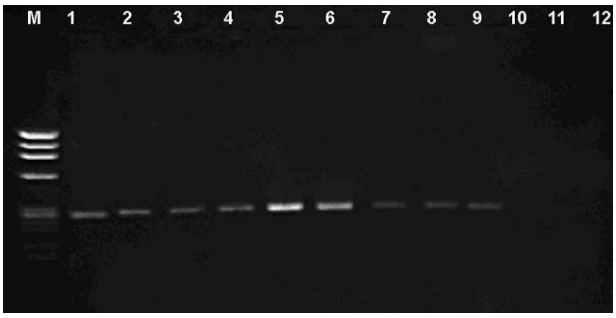

Fig. 1. The detection limit of HAV concentration in distilled water, as analyzed by agarose gel electrophoresis of amplification products. Lanes: (1) HAF-203; (2) HAF-203 diluted 1:10; (3) HAF-203 diluted $1:10^2$; (4) HAF-203 diluted $1:10^3$; (5) HAF-203 diluted $1:10^4$; (6) HAF-203 diluted $1:10^5$; (7) HAF-203 diluted $1:10^6$; (8) HAF-203 diluted $1:10^7$; (9) HAF-203 diluted $1:10^8$; (10) HAF-203 diluted $1:10^9$; (11) HAF-203 diluted $1:10^{10}$; (12) nested PCR negative control. *M*, 100 bp DNA ladder for molecular size standard.

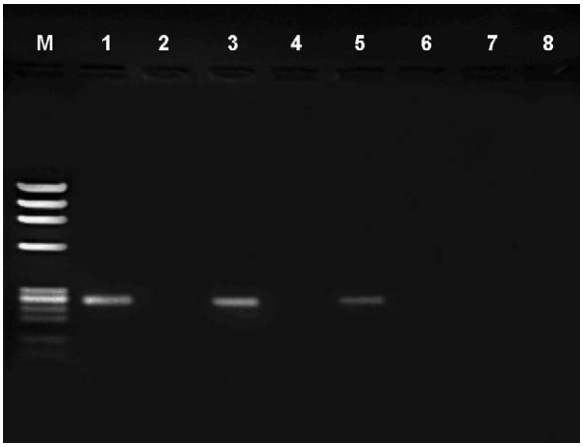

Fig. 2. Detection of HAV RNA in field water samples after concentration and using nested RT-PCR. Method 2 was used for HAV concentration in river and mineral water samples, and method 1 was used for coastal and tap water samples. Nested PCR products were visualized by agarose gel analysis and ethidium bromide staining. Lanes: (1) HAV-positive control of nested RT-PCR (247 bp); (2) coastal; (3) river; (4) mineral water; (5) tap water samples; (6–8) RT-PCR, PCR, and nested RT-PCR negative controls. *M*, 100 bp DNA ladder for molecular size standard.

samples, indicating that the detection limit remained the same as that seen for the concentration of HAV in distilled water.

3.3. Specificity and sensitivity of quantitative PCR

The HAV TaqMan® assay was evaluated for the detection of HAV RNA using the HAV strain HAF-203. The HAV was quantified in triplicate, and the detection limit was 60 genome/ml. The specificity of the assay was evaluated by testing the 10 non-

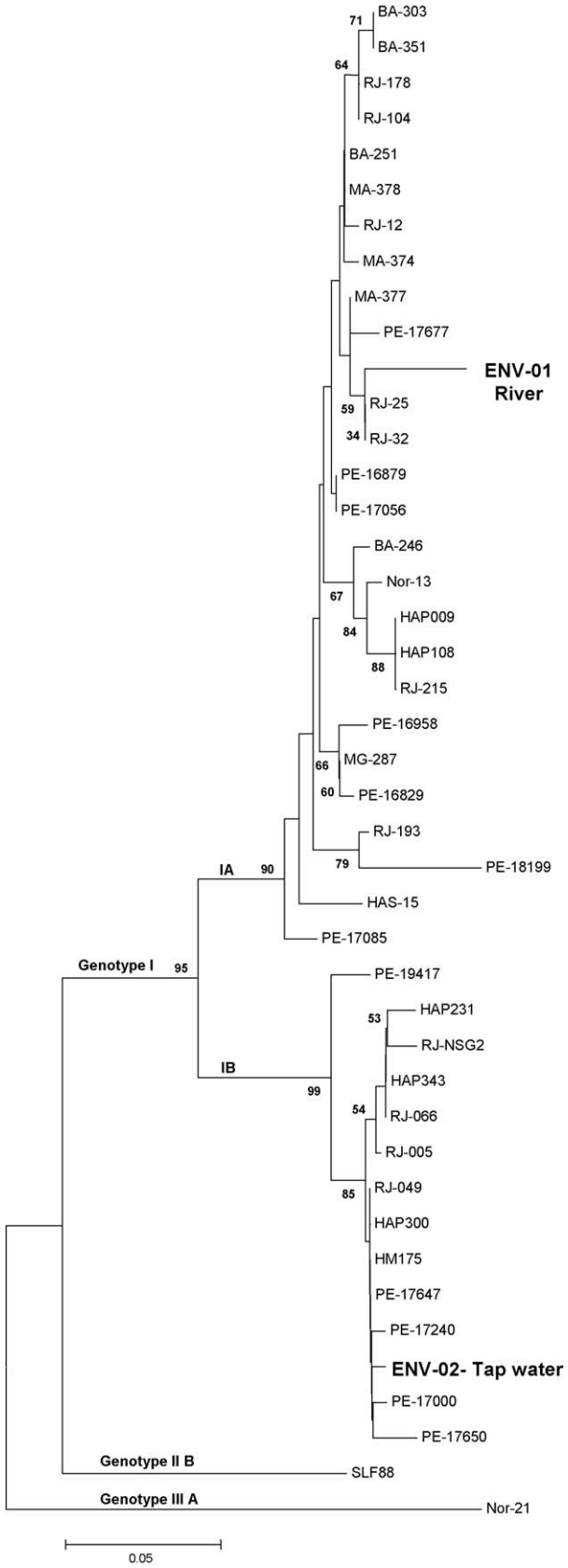

Fig. 3. Phylogenetic tree analysis, using the Kimura two-parameter model, of the VP1-2A region of HAV sequences isolated in this study and of 41 sequences obtained from the GenBank database. Numbers at nodes show bootstrap percentages obtained after sampling 1000 replicates. The bar indicates genetic distance. Genotypes and subgenotypes are indicated for each branch. Number of parentheses indicates the number of identical sequences. Strains belonging to this study begin with ENV.

HAV samples used in the section below and no false-positive amplification results were observed when testing the non-HAV samples using the real-time assay. In addition, the RJ-12 strain was detected using this technique, demonstrating the ability of the assay to detect other HAV strains.

3.4. Qualitative PCR in environmental water samples

Of each of the four types of water examined in this study (mineral, tap, river and coastal water), five samples were collected. Method 2 was used for mineral and river water, whereas method 1 for tap and coastal water samples to study the field application of these two methods for testing environmental samples. HAV RNA was detected in one of the five river water samples collected in the metropolitan region of the city of Rio de Janeiro and in one of the five tap water samples collected from a child care center were there had been an outbreak of hepatitis A. No amplification was detected in coastal or in mineral water samples (Fig. 2). Nested RT-PCR products were submitted to nucleotide sequencing in order to confirm the presence of HAV RNA and to carry out the genotyping.

3.5. Sequence analysis

A BLAST software evaluation of the 247 bp fragment indicated that the two samples (river and tap water) testing positive in the nested RT-PCR showed high homology (94–99.5%) to the HAV prototype strains HAS-15 and HM-175. Fig. 3 shows the phylogenetic tree, constructed by the neighbor-joining method, of the two positive samples, together with 37 samples selected at random from Brazilian patients, as well as reference sequences from the subgenotypes IA, IB, IIIA and IIB. The results of the phylogenetic analyses show that river sample belongs to subgenotype IA and that the tap water sample belongs to subgenotype IB.

Identity among subgenotype IA isolates, including the river water isolate, varied from 90.8% to 96.8%. Identity among subgenotype IB isolates, including the tap water isolate, varied from 97.2% to 99.5%. A comparison of the predicted amino acid sequences of the VP1/2A region from environmental isolates demonstrated an identity between these two strains of approximately 90.3% (results not shown).

4. Discussion

Worldwide, and especially in developing countries such as Brazil, HAV is of significant epidemiological importance. Although the prevalence of infection has diminished (Vitral et al., 1998), outbreaks of the infection continue to occur (Villar et al., 2002, 2004a). New and better methods for detecting HAV in environmental water samples will be needed since the virus persists in this milieu, and contaminated water may be the source of many of these outbreaks. Qualitative PCR is a suitable tool for detecting HAV in biological and environmental samples (De Paula et al., 2002, 2004b; Villar et al., 2004a; Pina et al., 2001; Vaidya et al., 2002; Brassard et al., 2005). A highly sensitive technique for virus detection is needed in order to ascertain the presence of the virus in environmental samples. Qualitative PCR and quantitative PCR to detect HAV RNA were used in this study. Both techniques presented high sensitivity and specificity, a finding that is in agreement with those of previous studies demonstrating the applicability of these techniques in detecting HAV in environmental samples (Brooks et al., 2005; Vaidya et al., 2002; Pina et al., 2001; Morace et al., 2002)

These results show that both HAV concentration methods can be used in conjunction with qualitative PCR to detect HAV in river water samples, although neither combination detected inoculated HAV in coastal water samples. Method 1 was most suitable for tap water samples, and method 2 was most suitable for mineral water samples. Different inhibitory compounds can be present depending on the type of water and can be eliminated according to the technique used. Therefore, the choice of concentration method influences the detection of HAV when qualitative PCR is used.

To date, three molecular methods of HAV quantitation have been described. The first uses a Taqman probe (Costa-Mattioli et al., 2002; Jothikumar et al., 2005), the second uses molecular beacons (Abd el-Galil et al., 2005) and the third uses SYBR Green real-time RT-PCR assay (Brooks et al., 2005). In the present study, the Taqman probe amplified all samples using both concentration methods. In addition, HAV was detected in concentrations of up to 60 genome/ml. indicating higher sensitivity than that previously reported by Brooks et al. (2005). However, the level of detection was low in coastal water samples, possibly because this type of sample contains inhibitory compounds. Furthermore, method 2 were unable to concentrate the virus in tap water samples, despite the fact that HAV had been detected in those same samples. These results suggest that since quantitative PCR detects low concentrations of genome per mililitre it is more suitable than qualitative PCR for the detection of HAV RNA in environmental samples.

4.1. Conclusions

It is concluded that quantitative PCR is capable of detecting HAV RNA in the four types of water samples tested and that the concentration method has little effect on HAV detection. However, when using qualitative PCR, concentration method appears to be influential since neither concentration method tested allowed HAV RNA to be detected in coastal samples. In addition, method 1 provided better results for tap water samples, whereas method 2 was more efficient for testing river and mineral water samples. These results demonstrate the impact of the concentration method for the detection of HAV RNA by qualitative PCR. This is likely attributable to the fact that qualitative PCR is more sensitive to inhibitors than is quantitative PCR. Using methods 1 or 2 to concentrate the virus, we were unable to detect HAV with qualitative PCR in water samples presenting concentrations lower than 2310 genome/ml.

Sequence analysis identified HAV in water samples collected in the field as subgenotype IA (in river water samples) and subgenotype IB (in tap water samples from the child care center),

demonstrating that these two subgenotypes are in co-circulation in Brazil. This is in agreement with the findings of previous studies of sera collected from patients with acute hepatitis (De Paula et al., 2002, 2004a; Devalle et al., 2003; Villar et al., 2004a). Therefore, it can be inferred that HAV strains detected in river water and tap water are of human origin and have been disseminated into the environment. The presence of two subgenotypes has been demonstrated in a previous study involving environmental samples (Pina et al., 2001).

The present study describes the detection of HAV in environmental samples in Brazil, showing that, although the incidence of infection is diminishing, HAV still persists in the environment. Monitoring the presence of virus is a critical component of the evaluation of the quality of environmental water.

Acknowledgments

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References

Abbaszadegan, M., Stewart, P., LeChevallier, M., 1999. A strategy for detection of viruses in groundwater by PCR. Appl. Environ. Microbiol. 65, 444–449.

Abd el-Galil, K.H., el-Sokkary, M.A., Kheira, S.M., Salazar, A.M., Yates, M.V., Chen, W., Mulchandani, A., 2005. Real-time nucleic acid sequence-based amplification assay for detection of hepatitis A virus. Appl. Environ. Microbiol. 71, 7113–7116.

Brassard, J., Seyer, K., Houde, A., Simard, C., Trottier, Y.-L., 2005. Concentration and detection of hepatitis A virus and rotavirus in spring water samples by reverse transcription PCR. J. Virol. Methods 123, 163–169.

Brooks, H.A., Gersberg, R.M., Dhar, A.K., 2005. Detection and quantification of hepatitis A virus in seawater via real-time PCR. J. Virol. Methods 127, 109–118.

Costa-Mattioli, M., Monpoeho, S., Nicand, E., Aleman, M.H., Billaudel, S., Ferre, V., 2002. Quantification and duration of viraemia during hepatitis A infection as determined by real-time RT-PCR. J. Viral Hepat. 9, 101–106.

Cuthbert, J.A., 2001. Hepatitis A: old and news. Clin. Microbiol. Rev. 14, 38–58.

De Paula, V.S., Baptista, M.L., Lampe, E., Niel, C., Gaspar, A.M., 2002. Characterization of hepatitis A virus isolates from subgenotypes IA and IB in Rio de Janeiro, Brazil. J. Med. Virol. 66, 22–27.

De Paula, V.S., Lu, L., Niel, C., Gaspar, A.M., Robertson, B.H., 2004a. Genetic analysis of hepatitis A virus isolates from Brazil. J. Med. Virol. 73, 378–383.

De Paula, V.S., Villar, L.M., Morais, L.M., Lewis-Ximenez, L.L., Niel, C., Gaspar, A.M.C., 2004b. Detection of hepatitis A virus RNA in serum during the window period of infection. J. Clin. Virol. 29, 254–259.

Devalle, S., de Paula, V.S., de Oliveira, J.M., Niel, C., Gaspar, A.M.C., 2003. Hepatitis A virus infection in hepatitis C Brazilian patients. J. Infection 47, 125–128.

Felsenstein, J., 1993. Phylogenetic Inference Package, Version 3.5. Department of Genetics, University of Washington, Coastalttle, WA.

Gaspar, A.M.C., Vitral, C.L., Yoshida, C.F.T., Schatzmayr, H.G., 1993. Fast and high growth of a Brazilian hepatitis A virus (HAF-203) in primate cell line. Brazil. J. Med. Biol. Res. 26, 203–206.

Jothikumar, N., Aparna, K., Kamatchiammal, S., Paulmurugan, R., Saravanadevi, S., Khanna, P., 1993. Detection of hepatitis E virus in raw and treated wastewater with the polymerase chain reaction. Appl. Environ. Microbiol. 59, 2558–2562.

Jothikumar, N., Cromeans, T.L., Sobsey, M.D., Robertson, B.H., 2005. Development and evaluation of a broadly reactive TaqMan assay for rapid detection of hepatitis A virus. Appl. Environ. Microbiol. 71, 3359–3363.

Katayama, H., Shimasaki, A., Ohgaki, S., 2002. Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater. Appl. Environ. Microbiol. 68, 1033–1039.

Kittigul, L., Khamoun, P., Sujirarat, D., Utrarachkij, F., Chitprom, K., Chaichantanakit, N., Vathanophas, K., 2001. An improved method for concentrating rotavirus from water samples. Mem. Inst. Oswaldo Cruz 96, 815–821.

Kittigul, L., Ekchaloemkieta, S., Utrarachkija, F., Siripanichgona, K., Sujiraratb, D., Pungchittona, S., Boonthumc, A., 2005. An efficient virus concentration method and RT-nested PCR for detection of rotaviruses in environmental water samples. J. Virol. Methods 124, 117–122.

Koff, R.S., 1998. Hepatitis A. Lancet 30, 1643–1649.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA 2: molecular evolutionary genetics analysis software. Bioinformatics 17, 1244–1245.

Lu, L., Ching, K.Z., de Paula, V.S., Nakano, T., Siegl, G., Weitz, M., Robertson, B.H., 2004. Characterization of the complete genome sequence of the genotype II hepatitis A virus (CF53/Berne isolate). J. Gen. Virol. 85, 2943–2952.

Morace, G., Aulicino, F.A., Angelozzi, C., Constanzo, L., Donadio, F., Rapicetta, M., 2002. Microbial quality of wastewater: detection of hepatitis A virus by reverse transcriptase-polymerase chain reaction. J. Appl. Microbiol. 92, 828–836.

Normann, A., Pfisterer-Hunt, M., Schade, S., Graff, J., Chaves, R.L., Crovari, P., Icardi, G., Flehmig, B., 1995. Molecular epidemiology of an outbreak of hepatitis A in Italy. J. Med. Virol. 47, 467–471.

Pina, S., Buti, M., Jardi, R., Clemente-Casares, P., Jofre, J., Girones, R., 2001. Genetic analysis of hepatitis A virus strains recovered from the environment and from patients with acute hepatitis. J. Gen. Virol. 82, 2955–2963.

Poovorawan, Y., Theamboonlers, A., Chongsrisawat, V., Jantaradsamee, P., Chutsirimongkol, S., Tangkijvanich, P., 2005. Clinical features and molecular characterization of hepatitis A virus outbreak in a child care center in Thailand. J. Clin. Virol. 32, 24–28.

Robertson, B.H., Jansen, R.W., Khanna, B., Totsuka, A., Nainan, O.V., Siegl, G., Widell, A., Margolis, H.S., Isomura, S., Ito, K., et al., 1992. Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. J. Gen. Virol. 73, 1365–1377.

Robertson, B.H., Averhoff, F., Cromeans, T.L., Han, X., Khoprasert, B., Nainan, O.V., Rosenberg, J., Paikoff, L., DeBess, E., Shapiro, C.N., Margolis, H.S., 2000. Genetic relatedness of hepatitis A virus isolates during a community-wide outbreak. J. Med. Virol. 62, 144–150.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Tanaka, J., 2000. Hepatitis A shifting epidemiology in Latin America. Vaccine 18 (Suppl. 1), S57–S60.

Taylor, M.B., Cox, N., Very, M.A., Grabow, W.O.K., 2001. The occurrence of hepatitis A and astroviruses in selected river and dam waters in South Africa. Water Res. 35, 2653–2660.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Vaidya, S.R., Chitambar, S.D., Arankalle, V.A., 2002. Polymerase chain reaction-based prevalence of hepatitis A, hepatitis E and TT viruses in sewage from an endemic area. J. Hepatol. 37, 131–136.

Villar, L.M., da Costa, M.C.E., de Paula, V.S., Gaspar, A.M.C., 2002. Hepatitis A outbreak in a public school in Rio de Janeiro, Brazil. Mem. Inst. Oswaldo Cruz 97, 301–305.

Villar, L.M., Meyer, A., Lampe, E., Gaspar, A.M.C., 2004a. Genetic variability of hepatitis A virus isolates in Rio de Janeiro: implications for the vaccination of school children. Braz. J. Med. Biol. Res. 37, 1779–1787.

Villar, L.M., Amado, L.A., Gaspar, A.M.C., 2004b. In situ enzyme immunoassay for titration of a Brazilian hepatitis A virus strain (HAF-203). Brazil. J. Med. Biol. Res. 37, 1023–1027.

Vitral, C.L., Yoshida, C.F.T., Lemos, E.R.S., Teixeira, C.S., Gaspar, A.M.C., 1998. Age-specific prevalence of antibodies to hepatitis A in children and adolescents from Rio de Janeiro, Brazil, 1978 and 1995. Relationship of prevalence to environmental factors. Mem. Inst. Oswaldo Cruz 93, 1–5.

4.3. ARTIGO 3

Diniz-Mendes L, Paula VS, Luz SL, Niel C (2008). **High prevalence of human Torque teno Virus in streams crossing the city of Manaus, Brazilian Amazon**. *J Appl Microbiol*, 105:51-58.

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ORIGINAL ARTICLE

High prevalence of human Torque teno virus in streams crossing the city of Manaus, Brazilian Amazon

L. Diniz-Mendes[1], V.S. de Paula[2], S.L.B. Luz[3] and C. Niel[1]

1 Laboratory of Molecular Virology, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, Brazil
2 Laboratory of Technological Development, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, Brazil
3 Biodiversity in Health, Leônidas e Maria Deane Research Center, FIOCRUZ, Manaus, Brazil

Keywords
Anellovirus, nucleotide sequencing, real-time PCR, river water, Torque teno virus, viral indicator.

Correspondence
Christian Niel, Laboratory of Molecular Virology, Oswaldo Cruz Institute, FIOCRUZ, Avenida Brasil 4365, 21040-900 Rio de Janeiro, RJ, Brazil. E-mail: niel@ioc.fiocruz.br

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Abstract

Aims: Torque teno virus (TTV) is a human DNA virus chronically infecting most healthy individuals worldwide and can be transmitted by faecal–oral route. The occurrence of TTV was evaluated in the streams crossing the city of Manaus (Brazilian Amazon) over a 1-year period, four times a year.

Methods and Results: Fifty-two water samples were collected from 13 different locations. Viruses were concentrated from two litres of water by adsorption to negative membrane filters followed by ultrafiltration. TTV DNA was detected by PCR assays designed to detect all five TTV genomic groups. By conventional PCR, 19/52 (37%) samples were positive. By real-time PCR, TTV DNA could be detected in 48/52 (92%) samples. Viral loads ranged from 1300 to 746 000 genome equivalent per 100 ml of river water. Eleven distinct nucleotide sequences were obtained.

Conclusions: Our results show the wide distribution and diversity of TTV among Manaus urban micro basins.

Significance and Impact of the Study: The data presented here may contribute to substantiate TTV as a sensitive indicator of human contamination.

Introduction

Freshwater is emerging as one of the most critical natural resource issues facing humanity. High levels of water pollution, especially by sewage, are correlated to an increase in public health problems. The World Health Organization (WHO) estimated that four billion cases of diarrhoea per year, among other diseases, are associated with the lack of access to clean water (Kosek *et al.* 2003). As many cases are not reported, the true extent of the waterborne diseases is unknown. Current standards for the evaluation of the public health safety of recreational and consumption waters are mainly based upon bacteriological analysis. Total and faecal coliform counts are the most commonly used indicators for microbiological quality of water and human health risk assessment.

Human viruses are present in water contaminated by sewage. Large quantities of viruses are excreted in human faeces (and, eventually, in urine), which can cause illnesses when ingested, even at low concentrations. These diseases include paralysis, meningitis, epidemic vomiting and diarrhoea, myocarditis, congenital heart anomalies, infectious hepatitis and eye infections (WHO Report 1979). As viruses are important waterborne pathogens, it is essential to accumulate information on the nature and extent of viral contamination of water. Furthermore, concentrations of bacteria and viruses are not necessarily correlated (Jiang and Chu 2004). Virus detection in environmental and drinking water samples is therefore greatly relevant, notably in the developing countries, for the adoption of appropriate measures of prevention of waterborne viral outbreaks. Bacteriophages, polioviruses, hepatitis A virus (HAV), enteroviruses and adenoviruses, have been proposed as viral indicators of human waste pollution (Pina *et al.* 1998; Jiang and Chu 2004; Gregory *et al.* 2006).

Torque teno virus (TTV) is a small, non-enveloped virus, with a circular, single-stranded DNA genome of

3·7–3·9 kb in length. TTV has been associated with hepatic and pulmonary diseases in addition to haematologic disorders (see Hino and Miyata 2007 for review). It is now known however that TTV, provisionally classified in the floating genus Anellovirus, is a ubiquitous virus infecting 60–100% of the healthy individuals worldwide. These people are frequently infected with more than one TTV genotype. Indeed, TTV is a virus showing a great genetic diversity. TTV isolates have been classified into five major genogroups 1–5 (Peng *et al.* 2002). Faeces of viremic individuals contain TTV (Okamoto *et al.* 1998; Itoh *et al.* 2000), suggesting that the faecal–oral route is a common route of transmission. Owing to the high proportion of virus carriers in the general population and the putative high resistance of TTV to physicochemical agents, one can expect a high level of TTV contamination in the human environment (Bendinelli *et al.* 2001), particularly in the regions of the world where sanitation levels are low. In this context, TTV might be a useful marker of contamination for viruses of faecal origin.

The objective of this study was to detect human TTV and to measure its load in the streams crossing the city of Manaus, central Amazon, Brazil.

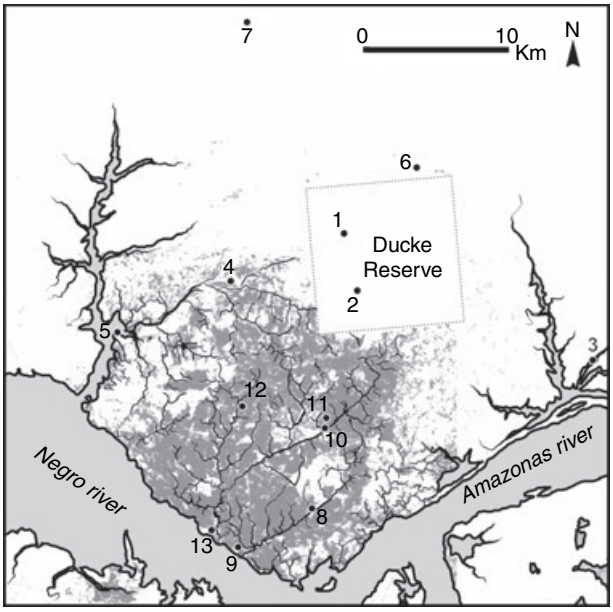

Figure 1 Satellite image of Manaus region (urban zone in grey) showing Negro and Amazonas rivers and Ducke biological reserve. Numbers 1 to 13 indicate the sites where water samples were collected. The exact localization of each site, determined by global positioning system, has been reported before (de Paula *et al.* 2007).

Materials and Methods

Water samples

Manaus is the main city of the Brazilian Amazon, with about 1·7 million inhabitants, i.e. about 10 times more than 30 years ago. Sewage collection and treatment is available for less than 10% of the population. A web of small rivers and streams that constitutes a draining system for rainwater and untreated sewage crosses the city. The waters of these small rivers are discharged into the Negro river.

Figure 1 shows the localization of the 13 sites where stream water samples were collected. Sites 1 and 2 were located in Ducke reserve, a biological reserve open to scientists and students for environmental observations and studies. Owing to horizontal expansion of the city, Ducke reserve is being submitted to a strong anthropic pressure. Site 3, accessible by boat, is surrounded by small riverine communities. Sites 4 to 7 were in rural areas, of low populational density. Sites 8 to 13 were located in the urban zone. At each site, four water samples were collected during 1 year, according to the pulse of main Negro river. Months of collection were August (ebb tide of Negro river) and November (low tide) 2004, in addition to February (flood tide) and June (high tide) 2005. Manaus is a city where rain falls almost every day along the year. However, the rain intensity is higher in November and February than in June and August.

Physicochemical analysis and coliform counts

Two litres of river water were collected in a sterile polycarbonate bottle (Nalgene, Rochester, NY) between 9:00 and 12:00 a.m. Temperature, dissolved oxygen, conductivity and pH were measured at sampling. Dissolved oxygen and conductivity were measured using a Model 85 digital meter (YSI, Yellow Springs, OH, USA). Samples were transported for 1–2 h in an ice-refrigerated container, until laboratory procedures start. Concentration of total and faecal coliforms were determined by the most probable number (MPN) method.

Virus concentration

Virus concentration was carried out using a previously described method (Katayama *et al.* 2002), with a few modifications. Briefly, $MgCl_2$ was added to water samples to a final concentration of 3 mmol l^{-1}, and the pH was adjusted to 5 with HCl, when necessary. Water samples were prefiltered through glass fibre AP20 (Millipore, Bedford, MA, USA) to remove suspended particulate matter, and filtered through a negatively charged, cellulose ester HA membrane of 0·45 μm pore size and 142 mm of diameter (HAWP14250, Millipore). These HA filters were then washed with 300 ml of 0·5 mmol l^{-1} H_2SO_4 (pH 3·0) to rinse out the cation

excess. Viruses were eluted with 15 ml of 1 mmol l^{-1} NaOH (pH 10·5–10·8). Fifty microlitres of 50 mmol l^{-1} H_2SO_4 and 50 μl of Tris-HCl 1 mol l^{-1}, pH 8·0, EDTA 100 mmol l^{-1}, were added to the eluate for neutralization. The eluate was submitted to ultrafiltration by centrifugation using a Centriprep YM-50 device (Millipore). Centrifugation was at 1500 ***g*** until reduction to a final volume of 2 ml (generally for 10–15 min).

DNA extraction and PCR

Viral DNA was extracted from 140 μl of the concentrate using a QIAamp DNA mini kit (Qiagen, Valencia, CA, USA), and resuspended in 50 μl of TE buffer (Tris 10 mmol l^{-1}, EDTA 1 mmol l^{-1}, pH 8). TTV DNA (3 μl) was amplified by a conventional, single-round PCR assay (Takahashi *et al.* 1998), able to detect TTV genotypes belonging to all the five genogroups. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light. TTV DNA concentrations were determined by TaqMan real-time PCR method, using sense TTVT1 (5′-TGCCGAAGGTGAGT TTACACA-3′) and antisense TTVT2 (5′-TTCAGAGCC-TTGCCCATAGC-3′) primers. Oligonucleotide probe was TTVTP (FAM-5′-CCCGAATTGCCCCTTGAC-3′-NFQ). Primers and probe were designed in the conserved, untranslated region of the genome, which allowed their annealing to DNA from human TTV isolates belonging to all genogroups. Amplicon size was 79 bp (nt positions 178–256 of TTV prototype TA278 isolate). Each reaction was performed in a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) with 3 μl of DNA, TaqMan universal PCR Master Mix (Applied Biosystems), 0·9 μmol l^{-1} of each primer and 0·25 μmol l^{-1} of probe for 45 cycles of two-temperature cycling (15 s at 95°C and 1 min at 60°C). To avoid false-negative results owing to the eventual presence of PCR inhibitors, all the PCR experiments were performed with both undiluted and 10-fold diluted DNA. Plasmid pCR4-TOPO (Invitrogen, Carlsbad, CA, USA), carrying the 79-bp amplicon, was used for standard curve setting. A strong linear relationship was observed between the amount of input plasmid DNA and the C_T values over seven log dilutions of the standard curve. Regression analysis of the C_T values generated by the log dilution series produced correlation coefficient values of 0·99. The slope of the regression curves indicated an increase of 3·42 cycles per log increase of DNA. Real-time PCR amplification efficiency (E), calculated from the regression analysis with the formula $E = 10^{-1/\text{slope}} - 1$, was 0·961. Sensitivity of the real-time PCR assay was 40 genome equivalent per reaction tube, corresponding to approximately 500 genome equivalent per 100 ml of river water.

Nucleotide sequence analysis

Amplicons from conventional PCR (199 bp) were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions, and cloned into PCR4-TOPO vector (Invitrogen) for nucleotide sequencing. This was performed by dye terminator reaction in both directions using M13 forward and reverse primers and dRhodamine terminator reagents. Reactions were analysed in an ABI Prism 3370 machine (Applied Biosystems). Sequences reported in this paper have been deposited in the GenBank database under the accession numbers EU118929 to EU118939. Alignments and phylogenetic analysis were performed using MEGA software, version 3.1 (Kumar *et al.* 2004). Phylogenetic tree was constructed by using the neighbour-joining method and Kimura two-parameter model.

Results

Physicochemical and bacteria indicators

Table 1 shows the physicochemical and bacteriological parameter values of the water samples. In all cases, the temperature of water was high (24·1–32·5°C) and stable (variations ≤5·2°C along the year at a given collect site). Mean dissolved oxygen values were 4·6, 5·2 and 1·2 mg l^{-1} for the sites located into the biological reserve (sites 1–3), rural area (4–7) and urban zone (8–13), respectively. Conductivity was much higher in the urban part (mean, 221 μs cm^{-1}) than in the rural (14 μs cm^{-1}) and biological reserve (9 μs cm^{-1}) areas. The pH values varied from about five (samples collected in the biological reserve) to seven (highly populated zone). In the reserve and rural areas, the mean total (3·0 log MPN 100 ml^{-1}) and faecal (2·3 log MPN 100 ml^{-1}) coliform counts slightly exceeded the international standards for recreational water [U.S. Environmental Protection Agency (EPA) 2002]. In the urban area however mean values were 100- to 1000-fold higher than the standards. Only small variations of coliform counts were observed according to the month of collect.

Presence of TTV DNA in water samples

By real-time PCR, TTV DNA was detected in 44/52 water samples when undiluted DNA aliquots were tested. Additionally, four negative samples became positive when 10-fold diluted DNA were used, probably owing to the effect of PCR inhibitors. At all, 48/52 (92%) water samples were positive (Table 2). Viral DNA loads of the positive

Table 1 Physicochemical and bacteriological parameter values of water samples collected in microbasins of Manaus

Parameter	Month of collect (2004/2005)			
	Aug	Nov	Feb	Jun
01				
Diss. oxygen (mg l^{-1})	6·1	3·0	6·6	6·9
pH	4·9	5·0	4·3	4·9
Conduct. (μs cm^{-1})	9·2	8·9	15·9	9·4
Temperature (°C)	24·7	24·9	25·3	25·6
TC (log MPN 100 ml^{-1})	3·8	4·2	3·1	3·0
FC (log MPN 100 ml^{-1})	3·3	4·1	≤2·3	2·8
02				
Diss. oxygen (mg l^{-1})	6·3	5·3	6·4	6·8
pH	4·5	4·6	4·8	4·9
Conduct. (μs cm^{-1})	15·9	12·7	18·8	13·5
Temperature (°C)	24·5	24·9	25·0	24·9
TC (log MPN 100 ml^{-1})	3·4	3·4	4·2	≤2·3
FC (log MPN 100 ml^{-1})	3·1	3·0	3·8	≤2·3
03				
Diss. oxygen (mg l^{-1})	0·7	5·4	4·3	4·0
pH	5·5	5·1	6·0	5·4
Conduct. (μs cm^{-1})	9·7	6·2	9·4	0·5
Temperature (°C)	28·0	31·5	26·3	30·1
TC (log MPN 100 ml^{-1})	≤2·3	2·6	2·8	2·8
FC (log MPN 100 ml^{-1})	≤2·3	2·6	≤2·3	2·8
04				
Diss. oxygen (mg l^{-1})	5·2	3·7	3·6	4·5
pH	7·4	6·2	6·7	6·4
Conduct. (μs cm^{-1})	32·6	43·7	49·6	36·6
Temperature (°C)	24·1	28·2	26·5	26·2
TC (log MPN 100 ml^{-1})	4·2	4·7	≥5·2	4·7
FC (log MPN 100 ml^{-1})	4·2	4·5	≥5·2	4·7
05				
Diss. oxygen (mg ml^{-1})	3·7	7·8	7·5	4·1
pH	5·3	7·8	6·8	5·2
Conduct. (μs cm^{-1})	10·1	50·6	10·5	10·8
Temperature (°C)	28·9	32·5	30·4	30·0
TC (log MPN 100 ml^{-1})	≤2·3	3·3	≤2·3	3·1
FC (log MPN 100 ml^{-1})	≤2·3	2·8	≤2·3	2·6
06				
Diss. oxygen (mg ml^{-1})	6·1	5·5	5·6	5·8
pH	5·2	5·2	4·4	5·0
Conduct. (μs cm^{-1})	6·6	6·4	15·2	7·8
Temperature (°C)	27·4	27·2	25·4	27·6
TC (log MPN 100 ml^{-1})	4·3	3·3	4·2	3·4
FC (log MPN 100 ml^{-1})	4·0	3·1	4·0	2·6
07				
Diss. oxygen (mg ml^{-1})	5·9	5·5	6·4	4·9
pH	5·6	5·3	5·1	5·6
Conduct. (μs cm^{-1})	7·3	7·0	7·4	5·5
Temperature (°C)	30·6	30·0	29·0	26·8
TC (log MPN 100 ml^{-1})	3·3	4·1	4·0	3·2
FC (log MPN 100 ml^{-1})	3·0	3·0	3·8	2·6
08				
Diss. oxygen (mg l^{-1})	4·0	0·5	2·1	1·5
pH	6·8	6·6	6·7	6·6
Conduct. (μs cm^{-1})	210·0	340·0	345·4	146·3
Temperature (°C)	27·7	28·8	27·7	27·5
TC (log MPN 100 ml^{-1})	≥5·2	≥5·2	≥5·2	≥5·2
FC (log MPN 100 ml^{-1})	≥5·2	≥5·2	≥5·2	≥5·2
09				
Diss. oxygen (mg l^{-1})	0·3	0·4	1·7	0·4
pH	6·7	6·9	6·5	6·6
Conduct. (μs cm^{-1})	210·0	351·1	124·9	66·9
Temperature (°C)	29·1	32·0	28·2	29·6
TC (log MPN 100 ml^{-1})	≥5·2	≥5·2	≥5·2	≥5·2
FC (log MPN 100 ml^{-1})	≥5·2	≥5·2	5·0	5·0
10				
Diss. oxygen (mg l^{-1})	3·1	0·5	2·1	0·5
pH	6·8	6·9	6·8	6·9
Conduct. (μs cm^{-1})	261·6	246·2	215·4	357·3
Temperature (°C)	30·0	30·0	27·5	30·9
TC (log MPN 100 ml^{-1})	≥5·2	≥5·2	≥5·2	≥5·2
FC (log MPN 100 ml^{-1})	≥5·2	≥5·2	≥5·2	≥5·2
11				
Diss. oxygen (mg l^{-1})	4·4	3·4	5·2	4·9
pH	6·9	7·1	6·9	7·0
Conduct. (μs cm^{-1})	182·2	289·3	176·4	179·7
Temperature (°C)	25·8	27·5	26·5	27·0
TC (log MPN 100 ml^{-1})	5·0	≥5·2	≥5·2	4·2
FC (log MPN 100 ml^{-1})	4·0	≥5·2	≥5·2	3·8
12				
Diss. oxygen (mg l^{-1})	2·3	1·1	3·1	2·4
pH	8·2	6·9	6·6	6·9
Conduct. (μs cm^{-1})	282·3	346·5	232·4	262·4
Temperature (°C)	27·7	29·6	26·5	27·0
TC (log MPN 100 ml^{-1})	≥5·2	4·2	≥5·2	≥5·2
FC (log MPN 100 ml^{-1})	≥5·2	≥5·2	5·0	≥5·2
13				
Diss. oxygen (mg l^{-1})	0·3	0·4	1·1	0·1
pH	6·5	6·9	6·8	6·7
Conduct. (μs cm^{-1})	169·0	418·5	221·2	113·5
Temperature (°C)	27·8	30·5	28·5	29·9
TC (log MPN 100 ml^{-1})	≥5·2	≥5·2	≥5·2	≥5·2
FC (log MPN 100 ml^{-1})	≥5·2	≥5·2	≥5·2	≥5·2

samples ranged from 1300 to 746 000 genome equivalent per 100 ml of river water. Geometric means of viral loads did not vary significantly according to the season (more or less rainy), pulse of inundation of Negro river, or geographic area (not shown). By conventional PCR, the presence of the virus was detected in 19/52 (36·5%) samples. No real-time PCR negative sample was positive by conventional PCR. Using this latter method, no negative sample became positive after 10-fold dilution. In 10/13 sites, at least one of the four samples collected was positive. The highest proportion of TTV positive samples (7/13, 54%) was observed in August, during the ebb of Negro river, whereas only 2/13 (15%) samples collected in February (flood tide, rainy season) were positive.

Table 2 Torque teno virus DNA detection by real-time PCR in micro basins crossing Manaus

Sample site	Month and year of collect			
	Aug 2004 (ebb tide, showers)	Nov 2004 (low tide, rainy season)	Feb 2005 (flood tide, rainy season)	Jun 2005 (high tide, showers)
01	48 600*	32 100	36 100	40 000*
02	58 200*	31 200*	38 900	17 500
03	2500*	3100*	3000	1600†
04	13 800	58 200*	<500	30 900†
05	56 400	26 300	46 100*	17 700
06	2100*	1300	27 600*	3100*
07	18 800*	66 100	65 000	54 300*
08	<500	746 300	47 800	49 000*
09	36 100	65 300	45 000	<500
10	70 000*	26 200*	20 500	83 900*
11	6000†	48 600	44 600	30 000
12	29 000*	62 800*†	45 300	30 900
13	13 500	23 700	<500	20 800

The results are expressed in genome equivalent per 100 ml of river water.

*Sample positive by conventional PCR.

†Sample negative when undiluted which became positive after a 10-fold dilution. For samples which were positive when both undiluted and 10-fold diluted, the highest value is shown.

Genetic variability of TTV in river water

The nucleotide sequences of the 19 amplification products obtained by conventional PCR were determined. Eleven distinct sequences were obtained (named Manaus 01 to 11). Figure 2 shows sequence alignment based on the 159 bp region analysed. TTV isolates showing identical sequences were found at different collect sites (sequence Manaus 01 at sites 10 and 12; Manaus 02 at sites 03 and 06; Manaus 03 at sites 01, 02 and 07; Manaus 08 at sites 02, 05 and 08; Manaus 10 at sites 06 and 10; Manaus 11 at sites 01 and 07). In the phylogenetic tree shown in Fig. 3, a cluster was composed by human TTV prototype isolate TA278 and the 11 sequences of this work. This cluster was phylogenetically distant from porcine, dog and cat TTV isolates. This confirmed that the sequences depicted in this work belonged to human, not animal virus.

Discussion

Water scarcity, pollution and management are the focus of a worldwide debate. Almost 50% of all people living in developing countries have at any given time a health problem caused by a lack of water and sanitation [United Nations Development Programme (UNDP) 2006]. Implicit in the use of faecal coliforms as indicator organisms has been the understanding that faeces of warm-blooded animals are the primary source of those bacteria in natural waters. Viruses have been shown to be more resistant than conventional indicator bacteria to wastewater treatment process and environmental degradation (Metcalf *et al.* 1995). Several viruses, such as adenoviruses (Jiang 2006), bacteriophages (Mandilara *et al.* 2006), HAV (Fernández-Molina *et al.* 2004) and polyomavirus (Hundesa *et al.* 2006) have been tested for their usefulness in indicating faecal pollution in environmental waters. Most enterically transmitted human viruses are excreted for

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Sequence     Site (month of collect)
                                        26       *         *         *         *         *         *         *       105
TA278                                   ACACCCACAGGCCAACCGAATGCTATGTCATCCATTTCCTGGGCCGGGTCTACGTCCTCATATAAGTAAGTGCACTTCCG
Manaus 01    10 (Aug), 12 (Aug)         ..T..TCTC......T.AG.GTT......G.G............AT.......A....G......AGCGC..........
Manaus 02    03 (Aug), 06 (Aug)         ..T...G.............GT...C...G.G..C........AG.A......A.GA.A......C.GGCA.T.......
Manaus 03    01 (Aug), 02 (Aug), 07 (Aug)  ..CT..G..........AT..........G.A..C.........T.T.........GA.A......A.GCT..........
Manaus 04    10 (Nov)                   ..T..............AG.GT.......G.G..C.........AT.........GA.A......AGCG...........
Manaus 05    12 (Nov)                   ..C...G..........AC.GT.......G.G..C.........AT.........GA.A......AGCG...........
Manaus 06    04 (Nov)                   ..T...G........T.AG.GT.......G.A..C.........AT............A......AGCG..A........
Manaus 07    03 (Nov)                   ..T..TCTC.C....T.AG.GTA..C...G..............AT.......A....G.........GC..........
Manaus 08    02 (Nov), 05 (Feb), 08 (Jun)  ..T..............AG.GT.......G.G..C.........AT.........GA.A......AGCG...........
Manaus 09    06 (Feb)                   ..T...G..........AG.GT...C...G.G..C.........AT.......A..A.A.......A.G.C..........
Manaus 10    06 (Jun), 10 (Jun)         ..T...G.............GT...C...G.G..C........AG.A......A.GA.A......C.GGCA.T.......
Manaus 11    01 (Jun), 07 (Jun)         ..T..TCTC......T.AG.GTT......G.G............AT.......A....G......AGCGC..........

                                        106      *         *         *         *         *         *         *       184
TA278                                   AATGGCTGAGTTTTCCACGCCCGTCCGCAGCGGTGAAGCCACGGAGGGAGATCTCCGCGTCCCGAGGGCGGGTGCCGAA
Manaus 01    10 (Aug), 12 (Aug)         ............................G...AGATC..G......AGCGATCGA.....................G.
Manaus 02    03 (Aug), 06 (Aug)         ............................A.AAAA.GC.AC....AAA.A...CG.....................G.
Manaus 03    01 (Aug), 02 (Aug), 07 (Aug)  ............ATGCT..........G.A.A/.G.A......CA..G....CGAA.................G.
Manaus 04    10 (Nov)                   ............................AGATC..A...A...AGCGATCGA.....................G.
Manaus 05    12 (Nov)                   ............................AGATC..G.......AGCGATCGA.....................G.
Manaus 06    04 (Nov)                   ..............................CAGCA..........T....C.....................G.
Manaus 07    03 (Nov)                   ............................G...AGATC..G...A...AGCGATCGA.....................G.
Manaus 08    02 (Nov), 05 (Feb), 08 (Jun)  ............................AGATC..A...A...AGCGATCGA.....................G.
Manaus 09    06 (Feb)                   ..............................AGA.C..................CT.....................G.
Manaus 10    06 (Jun), 10 (Jun)         ............................A.AAAA.GC.AC....AAA.A...CG.....................G.
Manaus 11    01 (Jun), 07 (Jun)         ............................G...AGATC..G.......AGCGATCGA.....................G.
```

Figure 2 Alignment of the Torque teno virus (TTV) nucleotide sequences from this study. Sequence of human TTV prototype isolate TA278 is at the top. Dots represent identity in comparison with the top sequence. The slash represents a 1-nt deletion.

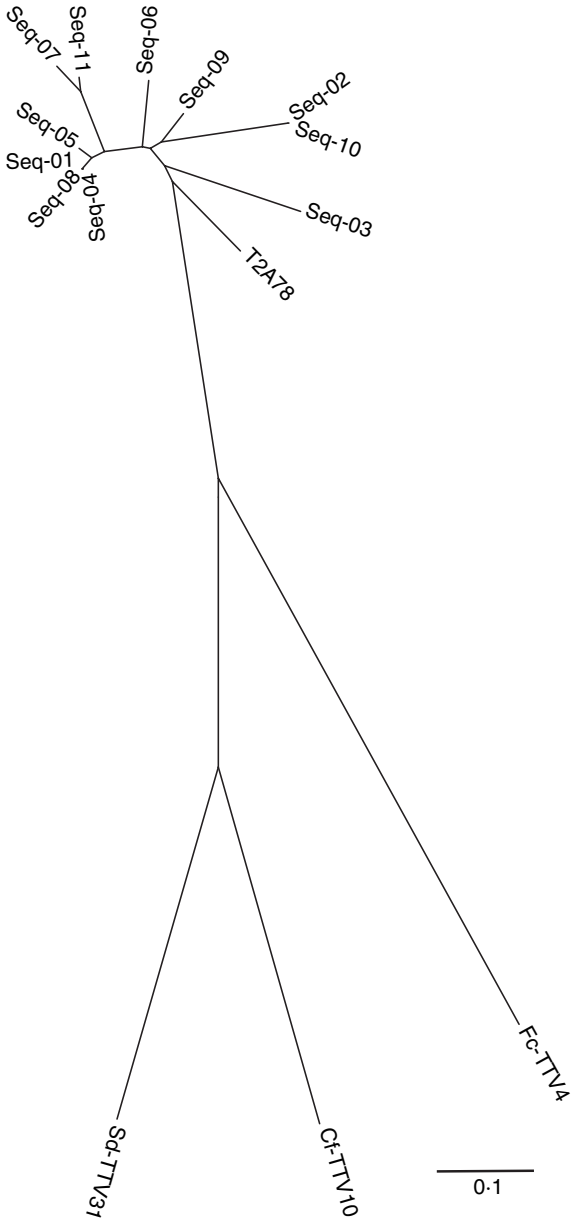

Figure 3 Phylogenetic tree (neighbour-joining method) constructed with sequences (nt positions 26–184) of human (prototype TA278), cat, dog and pig TTV (GenBank accession numbers AB017610, AB076017, AB076014, AB076006, respectively) in addition to sequences depicted in this work. Horizontal bar represents genetic distance.

short periods of time and during outbreaks. Differently, TTV is a widespread human DNA virus that produces persistent viremia and is excreted in the absence of overt clinical manifestations. These characteristics suggest that TTV should be a valuable indicator to evaluate human contamination from faecal origin. Recently, TTV DNA has been detected by PCR in environmental samples, such as sewage, river water and seawater (Vaidya *et al.* 2002; Haramoto *et al.* 2005a,b; Carducci *et al.* 2006; Verani *et al.* 2006). However, no data on the levels of TTV in environment were available until now.

In this work, the presence of TTV DNA was evaluated in streams crossing the county of Manaus, Central Amazon, Brazil, more precisely in areas of high, medium and low populational density. At nine out of 13 sites, the four samples collected in four different months of the year (August, November, February and June) were TTV DNA positive. At each of the remaining four sites, three samples were tested positive and one was negative. That positivity rate was similar to that recently reported for HAV in the same area (de Paula *et al.* 2007), which is highly endemic for hepatitis A. However, TTV and HAV loads did not show any significant correlation. In both cases, the same method of virus concentration (Katayama *et al.* 2002) was applied, with few modifications. This efficient technique has been shown to recover up to 95% of poliovirus particles from pure water and seawater. Although numbers of TTV genome equivalents may be very different from the numbers of infectious particles, the high rates of TTV positivity found here revealed a great dissemination of this virus in the streams and rivers crossing the Manaus county, including three sites belonging to a protected area (Ducke reserve). These data may contribute to substantiate TTV as a sensitive indicator of human faecal contamination.

Torque teno virus is a noncultivable virus whose presence can only be detected through PCR amplification of its DNA genome. However, environmental samples are prone to contain PCR inhibitors that may hamper amplification reaction (Fong and Lipp 2005). Here, no direct correlation was observed between TTV load and level of water pollution, as measured by physicochemical and bacteriological parameters. Furthermore, the fact that four samples giving negative results by real-time PCR became positive after a 10-fold dilution, strongly suggested the presence of PCR inhibitors. In this respect, it is noteworthy that Negro river is particularly rich in humic acid (Goulding *et al.* 1988), which is known to be a potent inhibitor of PCR.

The presence of some viruses in environmental samples may depend on the season or circumstances. For example, noroviruses and adenoviruses have been shown to be more frequent in winter and after rainfall events, respectively (Haramoto *et al.* 2006a,b). In the case of TTV, a virus that chronically infects the majority of humans, there is no reason to foresee any seasonality. Indeed, no correlation was observed between the TTV load and period of collect (Table 2). If confirmed, this absence of seasonality may be a positive factor for TTV

to be considered an indicator for contamination of human origin.

By phylogenetic analysis, it was demonstrated that all 19 characterized clones carried nucleotide sequences of human TTV (Fig. 3). Eleven distinct sequences were depicted, with three, five, two and three different sequences found in August, November, February and June, respectively. TTV is a virus showing an extensive genetic diversity (Peng *et al.* 2002; Hino and Miyata 2007). Although the small genomic region sequenced in this work did not allow to classify the isolates into genogroups and genotypes, it was possible to conclude that a large diversity of TTV isolates were circulating in the same city at the same time.

In conclusion, TTV DNA was detected at a very high frequency (92%) in the streams crossing the county of Manaus, Brazilian Amazon. At each collect site, including those located in rural area, at least three out of four samples, collected along 1 year, were TTV DNA positive. Among 19 TTV clones characterized, 11 distinct nucleotide sequences were depicted, showing the high genetic diversity of TTV isolates circulating at the same time in a geographically limited area. The data presented here suggest that TTV should be considered as a candidate indicator for contamination of human origin.

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References

Bendinelli, M., Pistello, M., Maggi, F., Fornai, C., Freer, G. and Vatteroni, M.L. (2001) Molecular properties, biology, and clinical implications of TT virus, a recently identified widespread infectious agent of humans. *Clin Microbiol Rev* **14**, 98–113.

Carducci, A., Verani, M., Battistini, R., Pizzi, F., Rovini, E., Andreoli, E. and Casini, B. (2006) Epidemiological surveillance of human enteric viruses by monitoring of different environmental matrices. *Water Sci Technol* **54**, 239–244.

Fernández-Molina, M.C., Álvarez, A. and Espigares, M. (2004) Presence of hepatitis A virus in water and its relationship with indicators of fecal contamination. *Water Air Soil Pollut* **159**, 197–208.

Fong, T.T. and Lipp, E.K. (2005) Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. *Microbiol Mol Biol Rev* **69**, 357–371.

Goulding, M., Carvalho, M.L. and Ferreira, E.G. (1988) *Rio Negro, rich life in poor water: Amazonian diversity and foodchain ecology as seen through fish communities*. The Hague, Netherlands: SPB Academic Pub.

Gregory, J.B., Litaker, R.W. and Noble, R.T. (2006) Rapid one-step quantitative reverse transcriptase PCR assay with competitive internal positive control for detection of enteroviruses in environmental samples. *Appl Environ Microbiol* **72**, 3960–3967.

Haramoto, E., Katayama, H., Oguma, K. and Ohgaki, S. (2005a) Application of cation-coated filter method to detection of noroviruses, enteroviruses, adenoviruses, and Torque teno viruses in the Tamagawa River in Japan. *Appl Environ Microbiol* **71**, 2403–2411.

Haramoto, E., Katayama, H., Oguma, K., Yamashita, H., Nakajima, E. and Ohgaki, S. (2005b) One-year monthly monitoring of Torque teno virus (TTV) in wastewater treatment plants in Japan. *Water Res* **39**, 2008–2013.

Haramoto, E., Katayama, H., Oguma, K., Yamashita, H., Tajima, A., Nakajima, H. and Ohgaki, S. (2006a) Seasonal profiles of human noroviruses and indicator bacteria in a wastewater treatment plant in Tokyo, Japan. *Water Sci Technol* **54**, 301–308.

Haramoto, E., Katayama, H., Oguma, K., Koibuchi, Y., Furumai, H. and Ohgaki, S. (2006b) Effects of rainfall on the occurrence of human adenoviruses, total coliforms, and *Escherichia coli* in seawater. *Water Sci Technol* **54**, 225–230.

Hino, S. and Miyata, H. (2007) Torque teno virus (TTV): current status. *Rev Med Virol* **17**, 45–57.

Hundesa, A., Maluquer de Motes, C., Bofill-Mas, S., Albinana-Gimenez, N. and Girones, R. (2006) Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment. *Appl Environ Microbiol* **72**, 7886–7893.

Itoh, Y., Takahashi, M., Fukuda, M., Shibayama, T., Ishikawa, T., Tsuda, F., Tanaka, T., Nishizawa, T. *et al.* (2000) Visualization of TT virus particles recovered from the sera and feces of infected humans. *Biochem Biophys Res Commun* **279**, 718–724.

Jiang, S.C. (2006) Human adenoviruses in water: occurrence and health implications: a critical review. *Environ Sci Technol* **40**, 7132–7140.

Jiang, S.C. and Chu, W. (2004) PCR detection of pathogenic viruses in southern California urban rivers. *J Appl Microbiol* **97**, 17–28.

Katayama, H., Shimasaki, A. and Ohgaki, S. (2002) Development of a virus concentration method and its application

to detection of enterovirus and Norwalk virus from coastal seawater. *Appl Environ Microbiol* **68**, 1033–1039.

Kosek, M., Bern, C. and Guerrant, R.L. (2003) The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. *Bull World Health Organ* **81**, 197–204.

Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Mandilara, G., Mavridou, A., Lambiri, M., Vatopoulos, A. and Rigas, F. (2006) The use of bacteriophages for monitoring the microbiological quality of sewage sludge. *Environ Technol* **27**, 367–375.

Metcalf, T.G., Melnick, J.L. and Estes, M.K. (1995) Environmental virology: from detection of virus in sewage and water by isolation to identification by molecular biology – a trip of over 50 years. *Annu Rev Microbiol* **49**, 461–487.

Okamoto, H., Akahane, Y., Ukita, M., Fukuda, M., Tsuda, F., Miyakawa, Y. and Mayumi, M. (1998) Fecal excretion of a nonenveloped DNA virus (TTV) associated with posttransfusion non A-G hepatitis. *J Med Virol* **56**, 128–132.

de Paula, V.S., Diniz-Mendes, L., Villar, L.M., Luz, S.L., Silva, L.A., Jesus, M.S., da Silva, N.M. and Gaspar, A.M. (2007) Hepatitis A virus in environmental water samples from the Amazon Basin. *Water Res* **41**, 1169–1176.

Peng, Y.H., Nishizawa, T., Takahashi, M., Ishikawa, T., Yoshikawa, A. and Okamoto, H. (2002) Analysis of the entire genomes of thirteen TT virus variants classifiable into the fourth and fifth genetic groups, isolated from viremic infants. *Arch Virol* **147**, 21–41.

Pina, S., Puig, M., Lucena, F., Jofre, J. and Girones, R. (1998) Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. *Appl Environ Microbiol* **64**, 3376–3382.

Takahashi, K., Hoshino, H., Ohta, Y., Yoshida, N. and Mishiro, S. (1998) Very high prevalence of TT virus (TTV) infection in general population of Japan revealed by a new set of PCR primers. *Hepatol Res* **12**, 233–239.

United Nations Development Programme (2006) Annual Report: Global partnership for development. http://www.undp.org/publications/annualreport2006/english-report.pdf

U.S. Environmental Protection Agency (2002) Implementation guidance for ambient water quality criteria for bacteria. http://www.epa.gov/waterscience/standards/bacteria/bacteria.pdf

Vaidya, S.R., Chitambar, S.D. and Arankalle, V.A. (2002) Polymerase chain reaction-based prevalence of hepatitis A, hepatitis E and TT viruses in sewage from an endemic area. *J Hepatol* **37**, 131–136.

Verani, M., Casini, B., Battistini, R., Pizzi, F., Rovini, E. and Carducci, A. (2006) One-year monthly monitoring of Torque teno virus (TTV) in river water in Italy. *Water Sci Technol* **54**, 191–195.

World Health Organization (1979) Human viruses in water, wastewater and soil. Report of a WHO Scientific Group. *World Health Orga Tech Rep Ser* **639**, 5–50.

4.4. ARTIGO 4

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Hepatitis A virus in environmental water samples from the Amazon Basin ☆

V.S. De Paula[a,b,*], L. Diniz-Mendes[a], L.M. Villar[a], S.L.B. Luz[b], L.A. Silva[b], M.S. Jesus[b], N.M.V.S. da Silva[b], A.M.C. Gaspar[a]

[a]Department of Virology, Oswaldo Cruz Institute, Fiocruz/ Rio de Janeiro, Brazil
[b]Department of Biodiversity, Centro de Pesquisa Leônidas e Maria Deane/Fiocruz, Manaus, Brazil

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Hepatitis A virus (HAV) is a significant waterborne human pathogen. Of the global supply of potable water, Brazil retains 13%, of which 75% resides in the Amazon Basin. Although hepatitis A morbidity has declined progressively in Brazil as a whole, it remains high in the Amazon region. We used nested and real-time reverse-transcription polymerase chain reaction (RT–PCR) to detect and quantify the viral load in water samples from the Amazon Basin. Most samples tested positive (92%), with viral loads varying from 60 to 5500 copies /L, depending on sanitary conditions and the degree of flooding. Nested RT–PCR of the VP1-2A region detected HAV RNA in 23% of the samples. In low viral load samples, HAV was detected only with real-time RT–PCR, suggesting that this technique is useful for monitoring HAV contamination. The presence of HAV in water samples constitutes a serious public health problem.

1. Introduction

Water scarcity has been under intense discussion worldwide. According to data from the International Water Management Institute, 1.8 billion people will be living in countries or regions with absolute water scarcity by 2025. Brazil retains 13% of the global supply of potable water, and 70% of the potable water in Brazil is found in the Amazon Basin. Therefore, there is a great need for studies that monitor the quality of the water as well as for policies and investments that focus on sanitation.

The use of indicators to detect human pathogenic viruses in water quality research has been an area of considerable debate (Lees, 2000). Numerous outbreaks have made it clear that the presence of bacterial indicators of fecal contamination does not consistently correlate with the levels of pathogenic viruses. Some enteric viruses grow poorly in cell culture, which constitutes a problem when investigating strategies for virus control and prevention (Atmar and Estes, 2001). Current methods of detecting such viruses in environmental water samples rely on genome amplification using molecular techniques such as qualitative and quantitative real-time reverse-transcription polymerase chain reaction (RT–PCR) (Heim et al., 2003; Monpoeho et al., 2002; Jothikumar et al., 2005). Worldwide, virus detection in environmental and potable water samples is becoming an important strategy for

☆Sequences reported in this paper have been deposited in the GenBank database under the accession numbers DQ386265 and DQ386276.

*Corresponding author. Department of Virology, Oswaldo Cruz Institute, FIOCRUZ, Avenida Brazil 4365, 21045-900, Rio de Janeiro, RJ, Brazil. Tel.: +55 21 25984483; fax: +55 21 26706397.
E-mail address: vdepaula@ioc.fiocruz.br (V.S. De Paula).

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preventing outbreaks of infection with waterborne viruses. Water, sanitation and health are closely interrelated.

Hepatitis A virus (HAV), a member of the family Picornaviridae, is a nonenveloped virus with a linear, single-stranded, positive-sense RNA genome of approximately 7.5 kb in length. The most common vehicles for HAV transmission are ingestion of contaminated water, consumption of contaminated foods and contact with infected persons. There is only one HAV serotype, which is organized into six genotypes (I–VI) (Lu et al., 2004; Robertson et al., 1992; Costa-Mattioli et al., 2003). Due to the occurrence of various outbreaks and isolated cases, HAV is currently recognized as a significant waterborne human pathogen. It is very stable in the environment (Sobsey et al., 1988) and has shown to be resistant under diverse environmental and substrate conditions (Zachoval and Deinhardt, 1993; Green and Lewis, 1999; Cuthbert 2001; Croci et al., 2002; Morace et al., 2002). In addition, it has been shown that, during food handling, more than 1000 virus particles can be transferred to the food from infected workers whose hands are contaminated with fecal matter (Bidawid et al., 2000). From 1980 to 2002, a progressive decline in the rate of hepatitis A morbidity was observed in all regions of Brazil. During that period, the morbidity rate was consistently higher in the north of Brazil (Vitral et al., 2006). In the Amazon region, a high prevalence of anti-HAV has been correlated with poor water quality and with poor sanitary conditions. In the city of Manaus, the water supply is provided by a microbasin network of springs and small rivers. This constitutes a draining system not only for water but also for sewage disposal. In the present study, microbiological and virological aspects were evaluated in water samples collected from 13 small rivers following four separate flooding events.

2. Materials and methods

2.1. Site description

The Brazilian Amazon has an area of over 2.7 million square miles. Though there are a few cities, the continental floodplain remains almost entirely covered with primitive tropical forest. Within this vast watershed, the Negro River, a tributary of the Amazon River that forms the northern part of the catchments basin, drains over 300,000 square miles, which represents 10% of the entire Amazon Basin. The water level in the Negro River fluctuates greatly, varying by as much as 15 m between the dry and rainy seasons. In the rainy season (April–October), the water level can rise to 8 m above sea level, flooding extensive areas. During such flooding, flow rates vary from 75,000 to 212,000 m^3/s. The abundance of water in the Amazon Basin is due to the fact that much of it lies in the region that is below the intertropical convergence zone, where rainfall is at its maximum. Depending on the season and on the quantity of organic material, acidity in the Negro River can vary from pH 3.6 to pH 5.8. At some points, the Negro river divides into main streams with inland and lateral channels, all connected by a network of natural canals and intricate channels, converging in function of the local topography.

2.2. Collection of environmental water samples

In this study, the presence of HAV was evaluated four times over a one-year period (August 2004–July 2005) in 13 streams that traverse the city of Manaus. The timing of the sample collection was based on the tidal stage (ebb, low, flood or high) of the main river (the Negro). A total of 52 water samples were analyzed, and the geological coordinates of sampling locations were determined using a global positioning system. Two-liter water samples were collected at 13 sites (Fig. 1), located in three different microbasins (Mindu, Tarumã and Quarenta). The sampling sites were selected based on differences in the extent of human settlement and on the degree of environmental impact on areas that include urban bayous. Two collection sites were located in the Ducke Forest Reserve, which is far from any urban areas and therefore assumed to be free of sewage.

All samples were collected in sterilized polypropylene carboys between 9:00 am and 12:00 am. Samples were transferred to 0 °C containers and delivered to the laboratory within 1–2 h of collection, at which point they were immediately processed. Physicochemical parameters such as conductivity, temperature and pH, as well as concentration of dissolved oxygen, were measured at each sampling site using a YSI 85 multiparameter sensor (YSI Environmental, Yellow Springs, OH, USA) and a pH sensor.

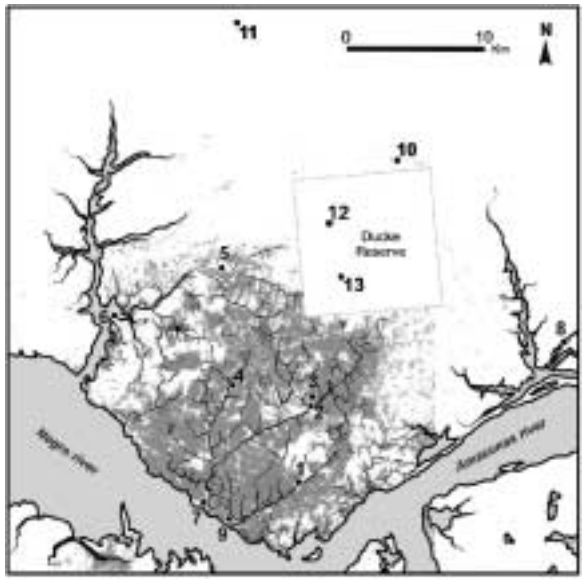

Fig. 1 – Manaus map showing 13 GPS points where samples were collected. Point 1—S 03°06′54,1″ W 059°58′15,0″; Point 2—S 03°03′48,1″ W 059°57′46,1″; Point 3—S 03°03′53.0″ W 059°57′44,9″; Point 4—S 03°03′04,4″ W 060°00′50,2″; Point 5—02°58′23,4″ 060°01′16,4″; Point 6—S 03°00′17,9″ W 060°05′29,4″; Point 7—S 03°07′41,5″ W 060°01′58,9″; Point 8—S 01°89′00,1″ W 096°65′74,2″; Point 9—S 03°08′19,5″ W 060°00′59,5″; Point 10—S 02°54′07,4″ W 059°54′25,2″; Point 11—S 02°45′47,7″ W 060°02′12,2″; Point 12—S 02°56′37,3″ W 059°57′03,6″; Point 13—S 02°58′42,6″ W 059°56′34,9″.

2.3. Analysis of coliforms

Total coliform and fecal coliform concentrations were analyzed by multiple tube fermentation and by the membrane filtration technique using standard methods (Clesceri et al., 1998).

2.4. Sample processing

The method used for determining HAV concentrations in water samples, was evaluated before and was most suitable for river water samples (Villar et al., 2006). This method was previously described by Katayama et al. (2000) and with the exception that we added 6 mM $MgCl_2$ and adjusted the pH to 5.0 with HCl. Water samples were filtered using a vacuum pump fitted with a type HA 0.45-μm negatively charged membrane filter (Millipore Corporation, Bedford, MA, USA). The filter was washed with 200 mL of 0.5 mM H_2SO_4 (pH 3.0) to remove cations, and the virus was eluted from the filter with 10 mL of elution buffer (1 mM NaOH; pH 10.8). The filter was recovered in a tube containing 50 μL of 100 mM H_2SO_4 (pH 1.0) and 100 μL of 100x Tris EDTA buffer (pH 8.0) for neutralization. The filtrate was then concentrated to 2 mL by centrifuging the samples in a Centriprep Concentrator (Millipore) at 1500 × g for 10 min. The samples contained a considerable amount of suspended particulate matter, which was processed both with and without filtering through an AP 20 membrane (retention rate, 0.8–8 μm) before applying it to the HA 0.45 μm negatively charged membrane filter, as described above.

2.5. RNA extraction and reverse transcription

Viral RNA was extracted from 140 μL of concentrated water samples and 60 μL of RNA were eluted using the commercial assay QIAamp Viral RNA (QIAGEN, Valencia, CA, USA). The cDNA was prepared at 37 °C over the course of 1 h using 10 μL of RNA, random primers (Life Technologies, Gaithersburg, MD, USA) and the Moloney murine leukemia virus reverse transcriptase (Life Technologies).

2.6. Detection of HAV by conventional nested RT–PCR and nucleotide sequencing

After reverse transcription, the VP1/2A junction region of the HAV genome was amplified using nested PCR as described elsewhere (de Paula et al., 2000). The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. Amplicons of the expected size (247 bp) were purified using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer instructions. Direct nucleotide sequencing was performed in both directions using dye terminator reaction with dRhodamine terminator reagents. Sense and anti-sense primers were used in the second round of PCR. Results were analyzed in an ABI Prism 3370 machine (Applied Biosystems, Foster City, CA, USA). Sequences reported in this paper have been deposited in the GenBank sequence database under the accession numbers DQ386265 and DQ386276.

2.7. Sequence analysis

The GCG package (Wisconsin Sequence Analysis Package, version 10.1; Genetic Computer Group, Madison, WI, USA) was used for nucleotide alignment and for establishing amino acid sequences. Multiple alignments were initially performed with the Clustal X program (Thompson et al., 1997). Further adjustments were performed manually using visual correction based on sequence comparison generated with the Chromas program (Conor McCarthy, Griffith University, Brisbane, Queensland, Australia). Phylogenetic trees were created using the neighbor-joining method and the Kimura two-parameter model (Felsenstein, 1993) with MEGA 2.1 software (Kumar et al., 2001). Phylogenetic tree reliability was assessed by bootstrap re-sampling (1000 pseudoreplicates).

2.8. Quantitative real-time RT–PCR

Standard curve: To evaluate the sensitivity of the quantitative real-time RT–PCR assay, HAV from cell culture (HAF-203 strain) was extracted using the reagent from the Viral QIA amp RNA extraction kit (QIAGEN). The RT–PCR was carried out as described previously (de Paula et al., 2002) using primers for the 5′ NC region (forward5′-TTTTCCCTTTCCTATTCCCTTTGT-3′ and reverse5′-AAAC-CATTCAACGCCGGAGGACT -3′). Both strands of HAF-203 were sequenced. The amplicon was then cloned into a TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) and a DNA plasmid from a recombinant clone was used for establishing standard curve points in real-time RT–PCR. The recombinant plasmid was quantified using a GeneQuant Spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA). Standard curves were generated using 10^2–10^7 copies of DNA plasmid. The standard curve was linear, with a slope of −3.49 ($R^2 = 0.998$). The real-time RT–PCR assay was able to detect as few as 60 copies/mL with a high degree of confidence. The probe and the primers were designed using Primer Express TaqMan gene expression assay (Applied Biosystems), based on the sequence of the cloned HAV. The forward primer (5′-CTGCAGGTTCAGGGTTCTTAAATC-3′) matches nucleotides 86 to 109 of the wild-type (M14707) sequence whereas the reverse primer (5′-GAGAGCCCTGGAAGAAAGAAGA-3′) matches nucleotides 219–240. The HAV-probe (FAM 5′-ACT-CATTTTTCACGCTTTCTG-3′) matches nucleotides 198–218.

2.9. Quantification with real-time PCR

All PCRs were performed using the 7500 Real-Time PCR System (Applied Biosystems). For each PCR run, a master mix was prepared at 0 °C with 1x TaqMan Universal PCR Master Mix (Roche, Nutley, NJ, USA) containing 1.25 μL of primers and probe (300 nM of each primer; 150 nM of probe; Gentec assay; Applied Biosystems). Five microliters of cDNA from water samples were diluted ten fold to prevent inhibitors from affecting the amplifications (Brooks et al., 2005; Jothikumar et al., 2005). One microliter of standard curve points was added to a PCR master mix of 20 μL. The thermal cycling conditions consisted of denaturation for

10 min at 94 °C followed by 40 cycles of 15-s at 94 °C and a final 1-min cycle at 60 °C.

2.10. Statistical analysis

Data are expressed as mean ± standard deviation. A simple linear regression model was used to determine whether the presence of HAV presented linear correlations with pH, concentration of dissolved oxygen, temperature, conductivity, fecal coliform counts or total coliform counts. A multiple logistic regression model was used to analyze which independent variable(s) significantly affected the detection of HAV. Differences were regarded as significant when a two-tailed $p<0.05$ was found. Analyses were made using the Statistical Package for the Social Sciences (SPSS for Windows, release 8.0; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Detection of hepatitis A virus

Qualitative and quantitative real-time RT–PCR assays were used to detect the presence of HAV in environmental samples from Manaus, the state capital of Amazonas. Using nested RT–PCR, we detected the presence of HAV in 23% (12/52) of the Negro River samples; four collected at ebb tide, three at low tide, one at flood tide, and three at high tide. When quantitative real-time RT–PCR was used, HAV was detected in 92% (48/52) of the samples. Four samples presented levels below the quantification limit and were therefore classified as being of undetermined status. In samples testing positive for HAV when quantitative real-time RT–PCR was used viral loads ranged from 60 to 5500 copies /L. All samples that tested positive for HAV in the nested RT–PCR presented viral loads higher than 1700 copies/L.

3.2. Parameters for monitoring water quality and for detecting HAV

Nested RT–PCR detection of HAV was found to correlate with sample viral load in a statistically significant manner ($p<0.001$). No correlation was found between HAV detection and the season at the time of collection. Total and fecal coliform counts were performed for all water samples. The total coliform counts for 67% (35/52) of the samples, as well as the fecal coliform counts for 73% (38/52), exceeded the World Health Organization guideline for potable water (5000 MPN/100 mL and 1000 MPN/100 mL respectively). In the 15 samples presenting fecal coliform counts ranging from 200 to 900 MPN/100 mL (permissible levels of bacterial contamination), the presence of HAV was not found to correlate with total or fecal coliform counts (Table 1). We also analyzed parameters such as pH, dissolved oxygen concentration,

Table 1 – Statistical correlations of parameters used to monitor water quality

Variable	Total (%)	Nested RT–PCR		Real-time PCR	
		Pos (%)	95% CI	Pos (> 60 copies) (%)	95% CI
PH			0.77–21.26		0.08–5.24
⩽6.0	42.3	2 (18.2)		20 (41.7)	
>6.0	57.7	9 (81.8)		28 (58.3)	
Dissolved oxygen concentration (%)			0.18–2.73		0.11–7.07
⩽4.0	51.9	6 (54.5)		25 (52.1)	
>4.0	48.1	5 (45.5)		23 (47.9)	
Temperature (°C)			0.25–4.02		0.07–4.24
⩽27	36.5	4 (36.4)		17 (35.4)	
>27	63.5	7(63.6)		31 (64.6)	
Conductivity (μs/cm)			0.39–0.76		0.02–3.16
⩽145	59.6	3 (27.3)		23 (47.9)	
>145	40.4	8 (72.7)		25 (52.1)	
Fecal coliform count (MPN/100 mL)			0.21–4.36		0.04–2.63
⩽1000	26.9	3 (27.3)		12 (25.0)	
>1000	73.1	8 (72.7)		36 (75.0)	
Total coliform count (MPN/100 mL)			0.31–6.04		0.05–3.54
⩽5000	32.7	3 (27.3)		15 (31.2)	
>5000	67.3	8 (72.7)		33 (68.8)	
Nested RT–PCR			—		0.00–4.94
Positive	23.0			12 (23.0)	
Negative	77.0			40 (77.0)	
Real-time RT–PCR (viral load)			0.00–4.85	—	
⩽60	8.0	0 (0)			
>60	92.0	12 (100)			

RT–PCR: reverse-transcription polymerase chain reaction; 95% CI: 95% confidence interval.

temperature and conductivity, which are used for monitoring water quality, but no statistically significant correlation was found between the presence of HAV and any of these parameters. Table 2 shows the physicochemical characteristics according to the principal Negro River fluctuations.

3.3. Sequencing

To investigate the genetic variability of HAV found in the water, we sequenced 220 nucleotides in the VP1/2A region. All strains fell into one of two clusters: subgenotype IA (nucleotide sequence diversity ranging from 1.4% to 5.2%); or subgenotype IB (nucleotide sequence diversity ranging from 2.8% to 4.7%). When HAV was detected during two different Negro River flood events were of the same subgenotype, with the exception of samples AM E-3 and AM H-3, which were collected at a site located near a hospital, co-circulation of the IA and IB subgenotypes was observed in samples collected, (Fig. 2) The molecular analysis of environmental samples and of previously sequenced serum samples collected in the Amazon (de Paula et al., 2006) indicated a relationship between the environmental and clinical samples (Fig. 2).

A phylogenetic tree based on 73 deduced amino acids revealed that nucleotide sequence heterogeneity in this region of the HAV genome results in only limited differences in the amino acid sequence (data not shown).

4. Discussion

The paucity of data regarding waterborne viruses makes it difficult to determine the risk they represent and precludes the development of plans to prevent viral transmission through contact with environmental water. In this study, we used two methods to evaluate HAV contamination of water in the Amazon Basin. Using nested RT–PCR, we detected HAV in 23% of the samples, compared with 92% using real-time PCR. This is likely attributable to the fact that qualitative PCR is more sensitive to inhibitors than is quantitative PCR (Villar et al., 2006). The real-time technique is more efficient in detecting HAV because it combines PCR amplification with the use of a probe to confirm the identity of the PCR product. It is also faster because there is no need for post-PCR confirmation of amplified products (Mackay, 2004). In addition, real-time RT–PCR is a powerful technique that is becoming increasingly more popular for rapidly detecting and quantifying pathogenic microorganisms in clinical and environmental samples (Brooks et al., 2005; Heim et al., 2003; Monpoeho et al., 2002; Jothikumar et al., 2005). The fact that the detection rate for nested RT–PCR was found to be lower than that seen for quantitative real-time RT–PCR is likely attributable to low viral loads and to the presence of inhibitors that can affect nested RT–PCR (Brooks et al., 2005). Two molecular methods of HAV quantitation in water samples have been described. The first uses a Taqman probe

Table 2 – Physicochemical characteristics according to the main Negro River tidal stage in the 52 samples collected

Tidal stage (no. of samples)	Temp (°C)	pH (µs/cm)	Conductivity (MPN/100 mL)	Total coliforms (MPN/100 mL)	Fecal coliforms	Dissolved oxygen concentration (%)	Viral load (copies/L)
Ebb tide (n = 13)							
Mean	27.4	6.1	108.0	73,938	72,361	47.4	6490
SD	2.0	1.1	110.0	76,302	76,678	28.4	10,404
Minimum	24.0	4.5	6.63	200	200	3.5	0
Maximum	30.6	8.3	282.2	160,000	160,000	79.3	36,670
Low tide (n = 13)							
Mean	29.0	6.1	163.6	69,538	77,500	49.0	10,381
SD	2.4	1.0	167.1	75,494	79,891	29.6	15,065
Minimum	24.0	4.6	6.17	400	4000	1.0	1660
Maximum	32.5	7.8	418.5	160,000	160,000	108.0	44,250
Flood tide (n = 13)							
Mean	27.14	6.1	110.0	89,784	80,200	55.1	5903
SD	1.59	0.9	115.05	79,112	75,691	27.9	11,323
Minimum	24.97	4.2	7.37	200	200	14.5	0
Maximum	30.43	6.9	345.4	160,000	160,000	101.8	42,600
High tide (n = 13)							
Mean	27.9	6.0	101	67,284	60,746	50.8	9810
SD	1.8	0.8	114.1	77,431	73,642	29.2	16,087
Minimum	24.9	4.9	50	200	200	1.0	0
Maximum	30.3	7.0	357.3	16,000	16,000	86.5	43,790

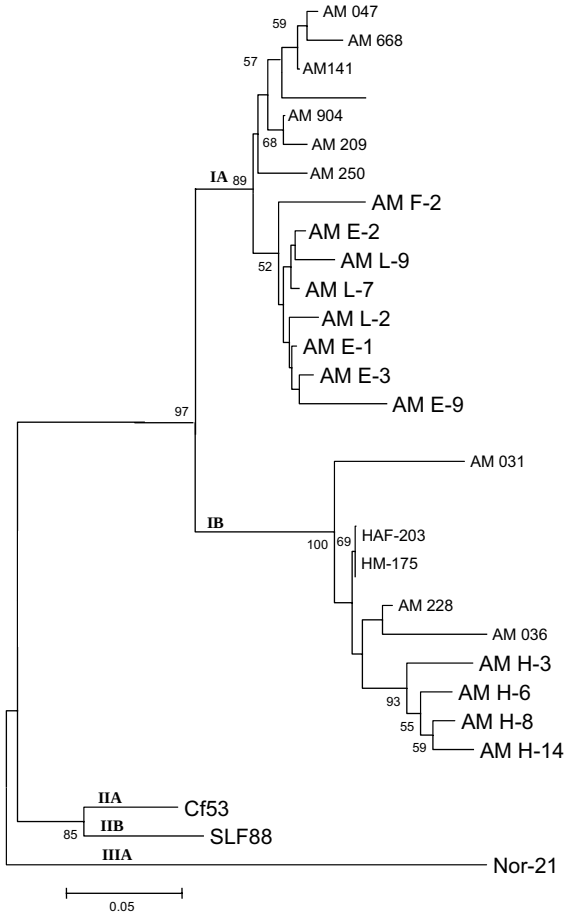

Fig. 2 – Phylogenetic tree based on 210-nt sequences of the VP1/2A region and depicting the genetic relatedness of isolates from this study (in bold) with other HAV strains previously characterized. The following abbreviations refer to Negro river tidal stage: E (ebb tide), L (low tide), F (flood tide) and H (high tide). The numbers at nodes indicate bootstrap percentages after the sampling of 1000 replicates. The horizontal bar provides a genetic distance scale.

(Jothikumar et al., 2005), the second uses SYBR Green real-time RT–PCR assay (Brooks et al., 2005). In the present study was used Taqman probe that in previously study was able to amplify HAV from tap, river, mineral and coastal water samples with concentrations of up to 60 genome equivalents/mL, indicating higher sensitivity than that previously reported using SYBR Green (Villar et al., 2006).

Although quantitative real-time RT–PCR is a rapid and sensitive technique that has been useful in detecting viruses in environmental samples presenting low viral loads, nested RT–PCR is still necessary for sequence analyses of the VP1/2A variable region (Nainan et al., 2006). The statistically significant correlation between detection by nested RT–PCR and sample viral load indicates that samples with low viral loads would only be detected through quantitative real-time RT–PCR.

In the northern part of Brazil, which includes the Amazon region, only 5.7% of households have sewage systems (Census 2000), which might explain the high frequency of HAV in environmental samples. Routine microbiological analyses do not identify viruses and use only bacterial parameters as indicators of contamination. It is known that HAV can survive for long periods in water and is not always accompanied by bacterial indicators (Soule et al., 1999). In general, waterborne viruses are more resistant to heat, disinfection and changes in pH than are bacteria. Numerous studies have addressed the stability of viruses under different circumstances. It has been shown that HAV is one of the most resistant enteric RNA viruses (Rzezutka and Cook, 2004). Because only a few viral particles are needed in order to cause disease, detection of minimal concentrations of the virus in water is important. Although it is not possible to determine whether the presence of HAV RNA leads to infection which is caused by virus particles it is likely that the RNA found in the water is accompanied by virus particles because the survival of naked RNA would be limited in an aquatic environment (Tsai et al., 1995). It is known that naked RNA does not bind to the membrane used for the selective recovery of viruses. Only viral capsids bind to the membrane while naked RNA runs through the columns during filtration. Any remaining RNA is rinsed out of the columns during the washing stage (Katayama et al., 2002).

No other seasonal effect was observed except for a minor peak in the detection curve of HAV at ebb tide. This could mean that untreated sewage is directly released in the environment. The high conductivity in samples collected at some sites might be related to discharge of pollutants into the rivers. Other physical parameters, such as dissolved oxygen concentration, temperature and pH, all of which are equally important in evaluating water quality, were analyzed. The presence of HAV was not found to correlate significantly with any of those parameters.

Sequence analysis of the VP1/2A region was chosen as a starting point because this region has proven useful in epidemiological studies (Robertson et al., 1992; Pina et al., 2001; de Paula et al., 2004). However, VP1 sequence information can also be used to determine genetic variability (Costa-Mattioli et al., 2002). In water samples testing positive for HAV, only genotype I was found. In clinical samples collected from Brazilian patients, this genotype is also predominant (de Paula et al., 2002, 2004; Villar et al., 2004), as it is in 80% of the strains seen worldwide. The fact that the environmental samples collected in the present study were found to belong to genotype IA or IB and presented, respectively, at least 95.2% and 97.4% homology with previously sequenced clinical samples collected from Brazilian patients (de Paula et al., 2006) suggests the existence of a link between the HAV found in the environment and that found in clinical samples. A less than 100% nucleotide identity between environmental and clinical samples has been observed (Pina et al., 2001). Two recent studies involving Amerindian and riverine communities in the Brazilian Amazon have shown that the endemicity of HAV is consistently high (93–98%) (de Paula et al., 2001; Nunes et al., 2004) In fact, our results lend support to the assumption that contaminated water serves as a source for the spread of HAV.

5. Conclusion

The fact that nested-PCR and real-time PCR both detected HAV in environmental water samples suggests that these

methods are more useful in evaluating the presence of viral contamination in such samples than are the parameters used to monitor water quality. The genetic homology found between clinical samples and water samples indicates that contaminated environmental water has the potential to cause and spread infection in communities, therefore representing a serious public health problem.

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REFERENCES

Atmar, R.L., Estes, M.K., 2001. Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. Clin. Microbiol. Rev. 14, 15–23.

Bidawid, S., Farber, J.M., Sattar, S.A., 2000. Contamination of foods by foodhandlers: experiments on Hepatitis A virus transfer to food and its interruption. Appl. Environ. Microbiol. 66, 2759–2763.

Brooks, H.A., Gersberg, R.M., Dhar, A.K., 2005. Detection and quantification of Hepatitis A virus in seawater via real-time RT–PCR. J. Virol. Methods 127 (2), 109–118.

Clesceri, L.S., Greenberg, A.E., Eaton, A.D., 1998. Standard methods for the examination of water and wastewater, 20th ed. American Water Works association, Washington, DC, 906pp.

Costa-Mattioli, M., Cristina, J., Romero, H., Perez-Bercof, R., Casane, D., Colina, R., Garcia, L., Vega, I., Glikman, G., Romanowsky, V., Castello, A., Nicand, E., Gassin, M., Billaudel, S., Ferre, V., 2002. Molecular evolution of hepatitis A virus: a new classification based on the complete VP1 protein. J. Virol. 76 (18), 9516–9525.

Costa-Mattioli, M., Di Napoli, A., Ferre, V., Billaudel, S., Perez-Bercoff, R., Cristina, J., 2003. Genetic variability of Hepatitis A virus. J. Gen. Virol. 84 (12), 3191–3201.

Croci, L., De Medici, D., Scalfaro, C., Fiore, A., Toti, L., 2002. The survival of Hepatitis A virus in fresh produce. International Journal of Food Microbiology 73, 29–34.

Cuthbert, J.A., 2001. Hepatitis A: old and new. Clin. Microbiol. Reviews 14, 38–58.

de Paula, V.S., Arruda, M.E., Vitral, C.L., Gaspar, A.M.C., 2001. Seroprevalence of viral hepatitis in riverine communities from Western region of the Brazilian Amazon Basin. Mem. Inst. Oswaldo Cruz 96, 1123–1128.

de Paula, V.S., Baptista, M.L., Lampe, E., Niel, C., Gaspar, A.M., 2002. Characterization of hepatitis A virus isolates from subgenotypes IA and IB in Rio de Janeiro, Brazil. J. Med. Virol. 66, 22–27.

de Paula, V.S., Lu, L., Niel, C., Gaspar, A.M., Robertson, B.H., 2004. Genetic analysis of Hepatitis A virus isolates from Brazil. J. Med. Virol. 73 (3), 378–383.

de Paula, V.S., Niel, C., Teves, S.C., Villar, L.M., Virgulino, H., Gaspar, A.M.C., 2006. Molecular epidemiology of Hepatitis A virus in Brazilian Amazon. J. Gastroenterol. Hepatol. 21, 1435–1438.

Felsenstein, J., 1993. Phylogenetic Inference Package, version 3.5. Department of Genetics, University of Washington, Seattle, WA.

Green, D.H., Lewis, G.D., 1999. Comparative detection of enteric viruses in wastewaters, sediments and oysters by reverse transcription-PCR and cell culture. Water Res. 33, 1195–1200.

Heim, A., Ebnet, C., Harste, G., Pring-Akerblom, P., 2003. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. J. Med. Virol. 70 (2), 228–239 J. Med. Virol. 2003. 71 (2), 320 (Erratum).

Jothikumar, N., Cromeans, T.L., Sobsey, M.D., Robertson, B.H., 2005. Development and evaluation of a broadly reactive TaqMan assay for rapid detection of Hepatitis A virus. Appl. Environ. Microbiol. 71 (6), 3359–3363.

Katayama, H., Shimasaki, A., Ohgaki, S., 2002. Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater. Appl. Environ. Microbiol. 68, 1033–1039.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA 2: molecular evolutionary genetics analysis software. Bioinformatics 17, 1244–1245.

Lees, D., 2000. Viruses and bivalve shellfish. Int. J. Food Microbiol. 59, 81–116.

Lu, L., Ching, K.Z., de Paula, V.S., Nakano, T., Siegl, G., Weitz, M., Robertson, B.H., 2004. Characterization of the complete genomic sequence of genotype II Hepatitis A virus (CF53/Berne isolate). J. Gen. Virol. 85 (10), 2943–2952.

Mackay, I.M., 2004. Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect. 10 (3), 190–212.

Monpoeho, S., Coste-Burel, M., Costa-Mattioli, M., Besse, B., Chomel, J.J., Billaudel, S., Ferre, V., 2002. Application of a real-time polymerase chain reaction with internal positive control for detection and quantification of enterovirus in cerebrospinal fluid. Eur. J. Clin. Microbiol. Infect. Dis. 21 (7), 532–536.

Morace, G., Aulicino, F.A., Angelozzi, C., Costanzo, L., Donadio, F., Rapicetta, M., 2002. Microbial quality of wastewater: detection of Hepatitis A virus by reverse transcriptase–polymerase chain reaction. J. Appl. Microbiol. 92 (5), 828–836.

Nainan, O.V., Xia, G., Vaughan, G., Margolis, H.S., 2006. Diagnosis of Hepatitis A virus infection: a molecular approach. Clin. Microbiol. Rev. 19 (1), 63–79.

Nunes, H.M., Soares Mdo, C., Silva, H.M., 2004. Infecção pelo vírus da hepatite A em área indígena da Amazônia oriental brasileira. Rev. Soc. Bras. Med. Trop. 37 (Suppl. 2), 52–56.

Pina, S., Buti, M., Jardi, R., Clemente-Casares, P., Jofre, J., Girones, R., 2001. Genetic analysis of hepatitis A virus strains recovered from the environment and from patients with acute hepatitis. J. Gen. Virol. 82 (12), 2955–2963.

Robertson, B.H., Jansen, R.W., Khanna, B., Totsuka, A., Nainan, O.V., Siegl, G., Widell, A., Margolis, H.S., Isomura, S., Ito, K., 1992. Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. J. Gen. Virol. 73 (6), 1365–1377.

Rzezutka, A., Cook, N., 2004. Survival of human enteric viruses in the environment and food. FEMS Microbiol. Rev. 28 (4), 441–453.

Sobsey, M.D., Shields, P.A., Hauchman, F.S., Davis, A.L., Rullman, V.A., Bosch, A., 1988. Survival and persistence of hepatitis A virus in environmental samples. In: Viral Hepatitis and Liver Diseases, New York, USA, pp. 121–124.

Soule, H., Genoulaz, O., Gratacap-Cavallier, B., Mallaret, M.R., Morand, P., Francois, P., Luu Duc Bin, D., Charvier, A., Bost-Bru, C., Seigneurin, J.M., 1999. Monitoring rotavirus environmental contamination in a pediatric unit using polymerase chain reaction. Infect. Control. Hosp. Epidemiol. 20 (6), 432–434.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Tsai, Y.L., Tran, B., Palmer, C.J., 1995. Analysis of viral RNA persistence in seawater by reverse transcriptase-PCR. Appl. Environ. Microbiol. 61 (1), 363–366.

Villar, L.M., Lampe, E., Meyer, A., Gaspar, A.M., 2004. Genetic variability of hepatitis A virus isolates in Rio de Janeiro: implications for the vaccination of school children. Braz. J. Med. Biol. Res. 37 (12), 1779–1787.

Villar, L.M., de Paula, V.S., Diniz-Mendes, L., Lampe, B., Gaspar, A.M., 2006. Evaluation of methods used to concentrate and detect hepatitis A virus in water samples. J Virol Methods, in press.

Vitral, C.L., Gaspar, A.M.C., Souto, F.J.D., 2006. Epidemiological pattern and mortality rate of Hepatitis A in Brazil, 1980–2002. Mem. Inst. Oswaldo Cruz. 101 (2), 119–127.

Zachoval, R., Deinhardt, F., 1993. Natural history and experimental models. In: Viral Hepatitis, London, UK, pp. 35–64.

4.5. ARTIGO 5

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Molecular Detection and Characterization of Gastroenteritis Viruses Occurring Naturally in the Stream Waters of Manaus, Central Amazônia, Brazil▽

Marize P. Miagostovich,[1] Fabiana F. M. Ferreira,[1] Flávia R. Guimarães,[1] Túlio M. Fumian,[1] Leonardo Diniz-Mendes,[1] Sérgio Luiz B. Luz,[2] Luciete A. Silva,[2] and José Paulo G. Leite[1]*

Laboratory of Comparative Virology, Oswaldo Cruz Institute, Rio de Janeiro,[1] and Biodiversity in Health, Leônidas and Maria Deane Research Center, Oswaldo Cruz Foundation (Fiocruz), Manaus,[2] Brazil

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To assess the presence of the four main viruses responsible for human acute gastroenteritis in a hydrographic network impacted by a disordered urbanization process, a 1-year study was performed involving water sample collection from streams in the hydrographic basin surrounding the city of Manaus, Amazonas, Brazil. Thirteen surface water sample collection sites, including different areas of human settlement characterized as urban, rural, and primary forest, located in the Tarumã-Açu, São Raimundo, Educandos, and Puraquequara microbasins, were defined with a global positioning system. At least one virus was detected in 59.6% (31/52) of the water samples analyzed, and rotavirus was the most frequent (44.2%), followed by human adenovirus (30.8%), human astrovirus (15.4%), and norovirus (5.8%). The viral contamination observed mainly in the urban streams reflected the presence of a local high-density population and indicated the gastroenteritis burden from pathogenic viruses in the water, principally due to recreational activities such as bathing. The presence of viral genomes in areas where fecal contamination was not demonstrated by bacterial indicators suggests prolonged virus persistence in aquatic environments and emphasizes the enteric virus group as the most reliable for environmental monitoring.

Although water is recognized as the most precious natural resource on our planet, human activities disregard this fact by continually polluting freshwater bodies. Increasing worldwide awareness of the poor quality of potable water has occurred mainly due to the significant increase in human morbidity and mortality. More than 2.2 million people die every year from diseases associated with poor quality water and sanitary conditions, mostly in developing countries. The presence of pathogenic enteric microorganisms in aquatic environments reveals how human health can be affected by contamination from sewage discharge into surface waters. It is estimated that nearly a quarter of all hospital beds in the world are occupied by patients presenting complications arising from infections caused by enteric microorganisms (53, 56).

Water sanitary quality is usually determined by the concentration of fecal indicator bacteria and occasionally by bacteriophages (8, 17). However, numerous investigations have shown that achieving minimum fecal coliform standards does not predict viral contamination (8, 47). Enteric viruses are highly stable in the environment, maintaining their infectivity even after exposure to treatment processes, and are often the most diluted pathogens in water, thus requiring concentration methods for their detection (2, 8, 42, 53).

After replication in the gastrointestinal tract, human enteric pathogenic viruses are excreted in high concentrations in the feces (10^5 to 10^{11}/g feces) and can enter the environment through the discharge of waste materials from symptomatic or asymptomatic carriers and therefore may be dispersed in environmental waters (2). Difficulties in obtaining viruses from environmental samples have been overcome through the association of virus concentration methods with the use of molecular techniques, such as PCR, which provide rapid, sensitive, and specific detection (2, 15, 16, 32, 35, 46, 48, 54).

Although the presence of viruses in water is underestimated, mainly due to the difficulties associated with the detection of such agents in different matrices, enteric viruses have been implicated in waterborne outbreaks in different countries every year (2, 36, 38, 53). Among these, rotaviruses (RV), noroviruses (NoV), human astroviruses (HAstV), and human adenoviruses (HAdV) are recognized as the most important etiologic agents of acute gastroenteritis and have been considered for environmental monitoring (11, 29, 55).

Diarrhea, a water-related disease, is a global public health problem and is ranked third among the causes of death affecting children under 5 years old, accounting for 17% of all deaths. It is estimated that 1.5 billion episodes occur each year, mostly in developing countries. It is recognized that a significant proportion of diarrhea cases caused by waterborne transmission in such countries is related to water quality. Levels of diarrhea disease differ between communities due to socioeconomic factors such as water availability and hygienic behavior (9, 45).

Despite a significant decrease in diarrhea-related mortality in developed and some developing countries, such as Brazil, diarrhea is still an important cause of morbidity in these coun-

* Corresponding author. Mailing address: Laboratório de Virologia Comparada, Pavilhão Cardoso Fontes, Av. Brasil 4.365, Manguinhos, CEP 21040-360, Rio de Janeiro, RJ, Brazil. Phone and fax: 5521-25602921. E-mail: jpgleite@ioc.fiocruz.br.

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tries (37). In the Northern region of Brazil, the city of Manaus reported an increase of 90.5% in the number of diarrhea cases between 1998 and 2000, from 8,878 cases to 16,914 (4).

The goal of this study was to assess viral contamination by the four main viruses responsible for acute gastroenteritis (RV, HAdV, HAstV, and NoV) in the hydrographic network that surrounds Manaus. Investigation and determination of the viruses that are dumped into streams from domestic sewage without prior treatment, as occurs in Manaus, could reveal how rapid population growth associated with a disordered urbanization process represents a threat to human health caused by the increased risk of disease transmission.

MATERIALS AND METHODS

Study area. The city of Manaus (3°S, 60°W) is the capital of Amazonas, which is the largest Brazilian state located in the northern region of the country. The city has an area of 11,458.5 km^2, corresponding to 0.73% of the territory of the State of Amazonas (1,577,820.2 km^2). The city of 1,403,796 inhabitants is located 900 miles (1,450 km) inland from the Atlantic coast, in the heart of the Amazon rain forest. It is surrounded by a dense hydrographic network composed by the Tarumã-Açu basin, which is partially situated within the urban area; the Puraquequara basin, which is located in the forest area; and the São Raimundo and Educandos microbasins of the Negro River basin, within the urban area of Manaus.

Thirteen precisely positioned sites located in these different basins were defined with a global positioning system (TREX Legend; Garmin Ltd., Olathe, KS). These sites were chosen for water sample collection and were positioned in different areas of human settlement; three control sites were represented by streams located in primary forest with intact vegetation and free of domestic sewage (sites 11 to 13); two sites were located at decamped areas, where the streams are also not affected by domestic sewage (sites 9 and 10); and eight sites were situated in regions of variable human settlement (sites 1 to 8). The São Raimundo and Educandos basins are characterized as urban areas with the presence of squatter slums without basic services such as sewage and a water supply. Some urban streams present different levels of water degradation processes, caused mainly by the complete or partial removal of riparian vegetation and by pollution from domestic sewage that is dumped into these streams without prior treatment (18).

Sampling schedule. The 1-year environmental surveillance was based on four sample collections at each site, conducted between August 2004 and June 2005, according to the annual fluctuation level of the Negro River, which usually shows a peak flood in June during the wet season. A total of 52 samples were obtained from collections performed in August (beginning of the dry season) and November (dry season), 2004, and in February (beginning of the wet season) and June (wet season), 2005.

Three-liter samples of surface water were collected in sterile bottles and transferred to the laboratory, where they were immediately stored at 4°C for viral and bacteria investigations, while the physicochemical parameters were measured in locum.

Physicochemical parameters. Temperature (°C), conductivity (μS), dissolved oxygen (DO; mg/liter), and pH were measured at the moment of collection with a YSI model 85 handheld salinity, conductivity, DO, and temperature system (YSI, Incorporated, Yellow Springs, OH) and a portable potentiometer (pH tester 2, waterproof, double junction).

Bacteriology. Standard multiple-tube fermentation and the membrane filtration technique for determining total and fecal coliforms were performed according to previously described protocols (17).

Virus concentration. The viral particles present in the samples were concentrated by the adsorption-elution method, with negatively charged membranes with the insertion of an acid rinse step for the removal of cations, as described previously (35). Briefly, the samples were prefiltered through an AP20 membrane (Millipore), and prior to process filtration, 1.2 g of $MgCl_2$ was added to 2 liters of water and the pH was adjusted to 5. The samples were filtered in a type HA negatively charged membrane (Millipore) with a 0.45-μm pore size with a vacuum pump system. The membrane was rinsed with 350 ml of 0.5 mM H_2SO_4 (pH 3.0), after which 15 ml of 1 mM NaOH (pH 10.8) was used to release the virus from the membrane. To neutralize the solution, 50 μl of 50 mM H_2SO_4 and 100× TE buffer (pH 8.0) was added. The eluate was filtered by using a Centriprep Concentrator 50 (Millipore) and centrifuged at 1,500 × *g* for 10 min at 4°C to obtain a final volume of 2 ml. The system was soaked briefly in a 10% bleach solution and rinsed in deionized H_2O prior to each use.

RNA and DNA extraction. Nucleic acid extraction was processed with the QIAamp viral RNA and QIAamp viral DNA kits (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

Reverse transcription (RT) reaction. cDNA synthesis was carried out by RT with a random primer (PdN_6; 50 A_{260} units; Amersham Biosciences, Chalfont St Giles, Buckinghamshire, United Kingdom) for three groups of enteric viruses, i.e., RV, HAstV, and NoV. Briefly, 2 μl of dimethyl sulfoxide (Sigma, St. Louis, MO) and 10 μl of RNA were mixed, heated at 97°C for 7 min, and chilled on ice for 2 min. The components of the mixture and their final concentrations for a 50-μl RT reaction were as follows: 2.5 mM each deoxynucleoside triphosphate (GIBCO BRL, Life Technologies, Inc., Grand Island, NY), 1.5 mM $MgCl_2$, 200 U of Superscript II reverse transcriptase (Invitrogen), and 1 μl of PdN_6. The RT reaction mixture was incubated in a thermal cycler (PTC-100 Programmable Thermal Controller; MJ Research, Inc., Watertown, MA) at 42°C for 60 min and 95°C for 10 min.

Primers and PCR protocols for virus detection. Primer characteristics and references for the amplification conditions of different PCR and nested PCR protocols used for nucleic acid detection of RV, NoV, HAstV, and HAdV were all described previously (3, 20, 24, 28, 43). To avoid false-positive results, quality control measures were followed as recommended and for each set of amplifications, negative and positive control samples were included. All methodologies were standardized with reference strains of each virus, and for the present study, previously characterized virus strains obtained from fecal samples were used as positive controls. The PCR products were resolved on 1.0% electrophoresis grade agarose gel (GIBCO BRL, Life Technologies, Inc., Grand Island, NY), followed by ethidium bromide staining (0.5 μg/ml), and images were obtained with the image capture system (BioImaging Systems) with the Labworks 4.0 software program.

Molecular characterization of RVs. Molecular characterization of RV was performed with specific primers routinely used for the binary classification of group A RV into G (VP7) and P (VP4) types, where G stands for glycoprotein and P stands for protease-sensitive protein. All procedures were performed with previously described primers and amplification conditions (20, 28).

Nucleotide sequencing of HAstVs, NoVs, and HAdVs. The amplicons of HAstV and NoV and the first round of HAdV (primers hex1deg and hex2deg) obtained in the PCR were sequenced to confirm the correct PCR products. The amplicons were purified with the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions and quantified by 1% agarose gel electrophoresis with the Low DNA Mass Ladder (Invitrogen) as a molecular pattern. The PCR amplicons were sequenced with an ABI Prism 3100 genetic analyzer and Big Dye Terminator cycle sequencing kit v. 3.1 (PE Applied Biosystems, Foster City, CA) in both directions, with the same primers used in the amplification reactions. CentriSep columns (Princeton Separations, Inc., Adelphia, NJ) were used to purify the sequencing reaction products, according to the manufacturer's recommendations.

Strain characterization and phylogenetic analysis. Nucleotide sequences were edited and aligned with the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The sequences were compared with their respective prototypes and to other sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=pubmed). Rooted phylogenetic trees were constructed with the MEGA 2 software (http://www.megasoftware.net/) by the neighbor-joining method, with genetic distance corrected by the Kimura two-parameter model with 1,000 pseudoreplicas.

Statistical analysis. Statistical results were produced by the software Epi Info, version 3.3, from the Centers for Disease Control and Prevention (http://www.cdc.gov/epiinfo/).

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences obtained in this study are DQ464891 to DQ464895 (HAdV), EF100670 (HAstV), and EF107526 (NoV).

RESULTS

Fifty-two river water concentrates were analyzed by PCR assay during this study, and at least one virus was detected in 31 (59.6%) of these. The number of viruses detected in water samples from different sites was variable (Table 1), totaling 50 virus strains, with RV (23 to 44.2%) verified as the most prevalent detected, followed by HAdV (16 to 30.8%), HAstV (8 to 15.4%), and NoV (3 to 5.8%). The different distributions of

TABLE 1. Sample collection site characteristics and virus groups detected in 52 surface water samples obtained during four collection periods

Human settlement level/ area type	Basin	Site no./stream	Virus(es) collected			
			August 2004	November 2004	February 2005	June 2005
Low/rural	Tarumã-Açú	1/estuary	HAstV	HAstV, RV	RV	
		2/medium	RV			RV
High/urban	São Raimundo	3/estuary	HAdV, RV	RV	RV	HAdV, HAstV, RV
		4/medium	HAstV, AdV	HAdV, HAstV, NoV	HAdV, HAstV, RV, NoV	HAdV, HAstV, RV
		5/medium		HAdV, RV	HAdV, HAstV, RV	HAdV
		6/medium		RV	RV	
High/urban	Educandos	7/estuary	HAdV	HAdV	NoV, RV	HAdV, RV
		8/medium	HAdV, RV	HAdV	HAdV, RV	RV
Low/rural	Puraquequara	9/decamped in 2001				RV
	Tarumã-Açú	10/decamped in 2002				
Very low/primary forest	Puraquequara	11/Mainã Grande		RV		
	Tarumã-Açú	12/Reserva Ducke	RV		RV	
		13/Reserva Ducke				HAdV

these viruses according to the collection area revealed that RV, HAstV, and HAdV were detected at sites located in the primary forest and rural areas (Table 2). The urban streams located at the Educandos (sites 7 and 8) and São Raimundo (sites 3 to 6) basins presented a high level of viral contamination, as revealed by the percentages of virus detection per site of 100.0% (8/8) and 81.3% (13/16), respectively. In these basins, all four groups of viruses investigated were detected, with HAdV and RV being the most frequent viruses present in the Educandos basin. Site 4, located in São Raimundo, presented the highest number of viral types detected and the highest variability of virus strains belonging to the four groups tested. This site is an urban stream presenting intact vegetation; however, it routinely receives contamination from sewage discharge originating from the surrounding houses.

The microbiological analysis results showed that the total and fecal coliform values in the samples exceeded those established by the standard methods used for the examination of water and wastewater guideline for recreational water (most-probable numbers [MPN], 5,000 and 1,000/100 ml) by 67.3% (35/52) and 73.1% (38/52), respectively. Sites that were positive for the presence of fecal coliforms included 6 of the 20 sites located in primary forest and rural areas, 4 out of 8 in the Tarumã-Açu microbasin, and all 14 sites located in the São Raimundo and Educandos microbasins. Only two sites (site 1, the Tarumã-Açu estuary, and site 12, the Ducke Forest Reserve) were negative for fecal contamination in the four water samples obtained during this study. However viruses were detected in the strains obtained from the estuary (Fig. 1). The Tarumã-Açu estuary (site 1) is perpendicular to the Negro River and has a few floating houses. The water is clear, besides being naturally dark and regularly used for recreational bathing.

TABLE 2. Frequencies of the viruses investigated and fecal coliforms detected in the areas studied

Pathogen	No. (%) found in:		Chi square	*P* value
	Urban area (n = 24)	Rural and forest areas (n = 28)		
Any enteric virus	21 (87.5)	10 (35.7)	14.4	<0.001
RV	15 (62.5)	8 (28.6)	6.0	0.014
NoV	3 (12.5)		3.7	0.09
HAstV	6 (25.0)	2 (7.1)	3.2	0.08
HAdV	15 (62.5)	1 (3.6)	21.1	<0.001
Fecal coliforms	24 (100.0)	14 (50.0)	16.4	<0.001

The frequency of sites where the presence of fecal coliforms exceeded an MPN of 1,000/ml and where virus detection was positive characterized significant microbiological contamination in the urban water streams and revealed that HAdV is the most significant marker of human presence (Table 2). Enteric virus detection in samples positive and negative for fecal coliforms was 63.2% (24/38) and 50.0% (7/14), respectively (P = 0.29), showing no correlation between these findings.

Global analysis for the presence of viruses and fecal coliforms by using recreational water parameters (MPN, 1,000/ml) according to the collection period demonstrated a slight increase in the number of strains detected during wet-season rainfall, although the number of positive sites for virus detection remained almost unaltered (data not shown).

The median values for the physicochemical parameters, including temperature, pH, DO, and conductivity, obtained at each site during the four sample collections are shown in Fig. 2. Observation revealed that the pH, DO, and conductivity generally followed two different patterns, characterizing the two distinct areas studied, i.e., areas with zero or minimal human settlement and areas where human activity clearly affected the local natural conditions. The former areas were characterized by acidic water presenting low electrical conductivity and high DO content. The urban areas (São Raimundo and Educandos) presented higher pH and conductivity values and low DO contents. The temperature of the water samples was the most stable parameter, ranging from 24.5°C to 30.6°C and from 24.0°C to 32.8°C in the respective areas.

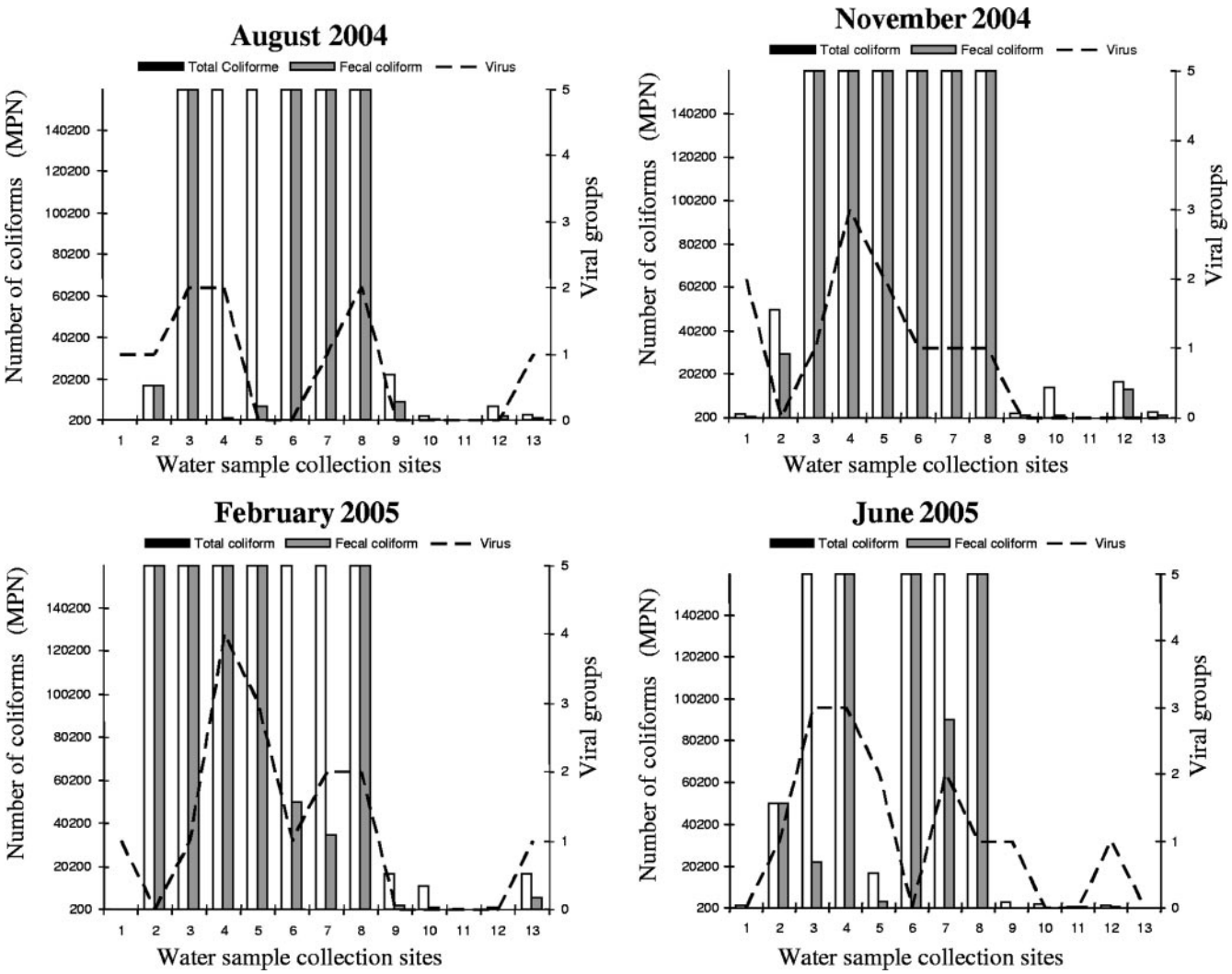

FIG. 1. Microbiological results obtained with water samples collected at 13 sites from streams of the Amazon hydrographic network according to the collection period.

Molecular characterization. Genotyping of VP4 of group A RV demonstrated that 13 samples were P[8], 1 was a coinfection of P[8] and P[4], and 1 was untypeable. Regarding VP7, 10 samples were characterized as G1 and 1 was untypeable. Only three samples were characterized for both VP4 and VP7 genes, and all of these were P[8],G1. Most of the positive samples were obtained from polluted streams; however, three were collected in rural and forest areas.

For HAdV, HAstV, and NoV genotyping, phylogenetic analysis was performed by comparing the nucleotide sequences obtained with their respective genotypes and with other strains available in the GenBank database representing different countries worldwide. For HAstV, the sequence analysis was performed based on a region of 348 nucleotides in open reading frame 2 of the HAstV genome, which clustered this strain with the HAstV Oxford type 1 prototype (nucleotide identity of 97.4%). Phylogenetic analysis performed to correlate the HAstV sequence with other isolates from different countries worldwide revealed a cluster including strains from Argentina, Colombia, and Brazil. Among the Brazilian strains, the environmental sequence displayed identities varying from 90.2% to 98.9%.

Partial sequencing of one strain was performed with region B primers to determine the NoV genogroups. Phylogenetic analysis revealed that this strain clustered within GII; however, we were unable to determine its genotype with this set of primers. Molecular characterization of HAdV was performed by sequencing 5 out of 16 detected strains. The sequence analysis corresponded to the 253 nucleotides between positions 47 and 299 within the hexon gene. The data generated were compared with the GenBank database and used to construct a phylogenetic tree, which, according to the nucleotide sequence identities (data not shown), showed that all of the strains belonged to species F; two of them were HAdV-40, and three were HAdV-41.

DISCUSSION

According to the Brazilian Institute of Geography and Statistics, Brazil has surface water resources of 168,870 m^3/s, rep-

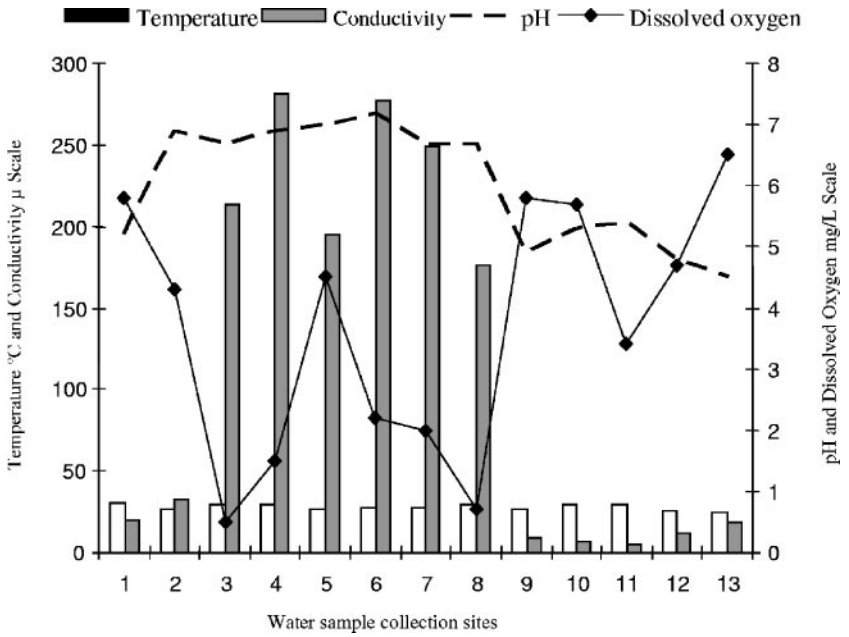

FIG. 2. Means of the physicochemical data obtained from the 52 surface water samples collected during the study period according to the collection sites.

resenting 50% and 11% of all the water available in Latin America and in the world, respectively. Seventy percent of Brazilian freshwater resources are located in the northern region of the country, in the Amazon hydrographic basin, where only 7% of the Brazilian population lives. Although this is a favorable situation, water quality is declining in most regions of the country, mainly due to rapid population growth without corresponding planning control. Similar to other Brazilian cities, Manaus has undergone rapid population growth in the last 3 decades, exhibiting growth from 300,000 inhabitants in the 1970s to 1,403,796 in the year 2000. Today, the urban population represents 99.4% of the total population (4, 39). This increase is the result of a large population influx to the city due to federal government policies that provide incentives for manufacturing activities initiated during the 1960s (12).

Urban development along the streams has led to a loss of biodiversity and to a decrease in water quality in these ecosystems, due to a series of adverse effects on these water bodies, including the discharge of untreated sewage directly into streams (12, 18, 41). The presence of the four main viruses responsible for acute gastroenteritis observed in the water of urban streams provides evidence that these viruses circulate at relatively high frequencies among the population of Manaus, while also reflecting the effects of increased population densities and anthropogenic activities on freshwater resources. In addition, this information could provide an assessment of the risk of human disease associated with sewage disposal into the streams of Manaus. These findings indicate the gastroenteritis burden of pathogenic viruses present in the water, due to the use of river water for drinking or due to other routes of transmission, such as poor hygiene, lack of sanitation, or even contamination due to recreational activities such as bathing in these areas. Studies showing viral diarrheal illness due to waterborne transmission related to poor water quality have been documented, including the ingestion of contaminated water during body contact recreation (22, 26, 31, 38, 52). In addition, the detection and characterization of HAdV, RV, HAstV, and NoV in environmental water samples may provide potentially useful data for epidemiological studies. The diversity of viruses detected in the water was variable and depends on factors like population density and infection prevalence within a given community, such that the amount of viruses dumped directly into river water in untreated discharge could explain the prevalence and distribution of such viruses.

In the present study, RV was the most prevalent virus detected, with P[8],G1 the only genotype characterized by a seminested, typing-specific PCR, a reflection within the environment concerning the impact of these viruses on the population. RV has been described as the most important virus in cases of acute gastroenteritis, since it is responsible for a third of these cases and RV P[8],G1 has been described as the most common genotype circulating worldwide (44, 49). In a study performed in Germany (47), RV RNA was detected and confirmed in 3 to 24% of the effluent and surface water samples tested.

The high percentage of RV detection in the present samples indicates that this virus should be considered for use as a potential indicator of fecal environmental contamination in developing countries, where their circulation in the environment appears to be higher than HAdVs, which are already considered a molecular index of human virus presence in the environment (7, 13, 46). Recently, an epidemiological surveillance of human enteric viruses of different environmental matrices detected the same viral strain in feces of gastroenteritis cases and in water and suggested both RV and HAdV as reference viruses for risk assessment (11). The stability of RV in environmental waters has been previously described, and its resistance to physiochemical treatment processes used by sewage treatment plants may facilitate its transmission (5).

Single-stranded RNA (ssRNA) virus detection rates were lower, especially for NoV. We believed that the protocol used could affect this result, and it is probable that the percentage of NoV detected would have been higher if a nested (or seminested) PCR assay had been used. The high sensitivity of the nested PCR has been described and for RV could detect double-stranded RNA from as few as 10 to 100 particles (28). Despite the low levels of NoV detected, the emergence of these viruses resulting in outbreaks of gastroenteritis is notable (6, 10, 33). To date, no reliable data regarding the frequency of NoV in Manaus are available and therefore the impact of these infections could not be measured. HAstVs have been recognized as important etiologic agents of viral gastroenteritis, contributing to 2 to 26% of the gastroenteritis cases in developing countries (19, 29, 55). The current rate of HAstV detection in this study is within the 6 to 50% range of surface water, as previously described (15, 29, 47).

The present results, based on direct molecular detection of viruses in river water, were confirmed by direct sequencing of PCR amplicons of one HAstV strain, one NoV strain, and six HAdV strains to ensure detection specificity. Nucleotide sequence analysis revealed the presence of HAstV-1 and NoV genogroup II strains, confirming the wide distribution of these viruses, as previously described (10, 14, 23, 25, 27, 33, 51). The fact that the HAdV detected belongs to species F, serotypes 40 and 41, was also verified. The different types of HAdV detected confirm that the quality control measures adopted throughout these procedures were sufficient to ensure these results. In addition, a study of hepatitis A virus (HAV) carried out with the same samples (21) corroborated these results.

The present study also evaluated the potential of the virus concentration method by negatively charged membrane filtration associated with different PCR protocols routinely used for stool samples. The detection of ssRNA, double-stranded RNA, and DNA viruses revealed that the association of the methods described here is a feasible approach for detecting the main waterborne enteric viruses responsible for gastroenteritis in environmental water samples collected from different grades of pollution. The lower virus detection rate in polluted water samples during the dry season could be explained by the concentration of inhibitors throughout this period. It has been demonstrated that the presence of organic compounds such as humic, fulvic, and tannic acids, proteins, and inorganic compounds such as metals present in the environment is a major obstacle to the routine detection of enteric viruses from environmental waters by PCR (1, 32, 50). In this study, 21 water samples were negative for the presence of HAdV, HAstV, NoV, and RV; however, considering that Torque teno virus and HAV were also investigated in the same water samples (results published elsewhere; 21; L. Diniz-Mendes, unpublished data), the number of water samples negative for all viruses was reduced to 11. Unfortunately, the presence of compounds that could inhibit RT-PCR/PCR was not evaluated. Previously, this method showed average recovery yields of spiked poliovirus of 62% from 1 liter of artificial seawater (35).

Recently, environmental virological studies have emerged worldwide and procedures for concentrating virus in water samples associated with different detection methodologies have been described (13, 30, 40, 48). In this study, we attempted to gain an initial insight into NoV, HAstV, RV, and HAdV occurrence within surface water samples of the Amazon basin. Thus, the PCR approach was useful as an alternative to overcome the limitations of conventional techniques, such as cell culture, since NoV cannot be grown in cell culture and RV, HAstV, HAdV-40, and HAdV-41 are fastidious agents (8, 15, 34). In fact, the characteristics of these viruses were determining factors when selecting the association of a membrane negative charged method with certain PCR methods, since conventional virus concentration procedures that use positive membrane and beef extract as an eluate are known to present certain inhibitory effects on PCR detection for viruses (1, 15, 46).

Although the method used for detecting enteric viruses cannot distinguish between infectious and noninfectious virions, the detection of an ssRNA genome in the environment suggests the presence of an infective virus since this molecule is not very stable under environmental conditions (40). Further studies concerning virus viability in these water samples should be performed by cell culture or cell culture associated with PCR, as previously described (15, 47).

The presence of viral genomes in areas showing low levels of fecal contamination by bacterial indicators suggests the prolonged persistence of these viruses in the environment and indicates that the enteric virus group is more reliable for environmental monitoring than bacterial indicators. It has been recognized that these viruses are more stable than bacteria in water and sewage, constituting not only a potential hazard but also good indicators of fecal pollution, as well as the potential presence of other viruses (8).

The anthropogenic influence on streams within the urban area of the municipality of Manaus was also notable due to the high quantities of total and fecal coliforms present in the water samples and the physicochemical analyses that corroborated previous findings, characterizing the São Raimundo and Educandos microbasins with an increased pH, high conductivity, and a low DO content (41). Monitoring of these streams revealed that viral contamination could be derived from infected residents of the São Raimundo and Educandos microbasins and reinforces the need to make improvements in water supply, sewage disposal, garbage collection, and urban drainage services, as suggested in previous reports (12, 41). The flooding events that occur annually in these areas may increase the number of waterborne disease exposure scenarios (4). Additionally, a previous study performed in the city of Manaus by using macroinvertebrates as bioindicators demonstrated that 80% of the streams within the urban area are impacted, such that their abiotic characteristics have been modified by deforestation and water pollution (18).

In Brazil, a lack of studies regarding viral contamination monitoring in surface water exists, and to the best of our knowledge, no investigation to date has evaluated the presence of viruses in river water of the hydrographic basin of Amazonas. Data concerning HAV obtained in the same study were published elsewhere (21). The viral contamination detected in this study provides a better assessment of human disease risk associated with sewage disposal into river water, increases our knowledge regarding this subject, and assists in the development of more efficient public health actions. The possibility of detecting human enteric viruses in a given water source will facilitate the provision of appropriate advice to public and

responsible authorities regarding the use and treatment of water. Continuous viral contamination monitoring is useful for preventing waterborne disease outbreaks and for understanding the impact caused by human occupation and the use of territories that contain freshwater resources.

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REFERENCES

1. **Abbaszadegan, M., M. S. Huber, C. P. Gerba, and I. L. Pepper.** 1993. Detection of enteroviruses in groundwater with the PCR. Appl. Environ. Microbiol. **59:**1318–1324.
2. **Abbaszadegan, M.** 2001. Advanced detection of viruses and protozoan parasites in water. Rev. Biol. Biotechnol. **2:**21–26.
3. **Allard, A., B. Albinsson, and G. Wadell.** 2001. Rapid typing of human adenoviruses by a general PCR combined with restriction endonuclease analysis. J. Clin. Microbiol. **39:**498–505.
4. **Anonymous.** 2002. Projeto geo cidades: relatório ambiental urbano integrado: informe GEO: Manaus. Consórcio Parceria, Rio de Janeiro, Brazil.
5. **Ansari, S. A., V. S. Springthorpe, and S. A. Sattar.** 1991. Survival and vehicular spread of human rotaviruses: possible relation to seasonality of outbreaks. Rev. Infect. Dis. **13:**448–461.
6. **Blanton, L. H., S. M. Adams, R. S. Beard, G. Wei, S. N. Bulens, M. A. Widdowson, R. I. Glass, and S. S. Monroe.** 2006. Molecular and epidemiologic trends of caliciviruses associated with outbreaks of acute gastroenteritis in the United States, 2000-2004. J. Infect. Dis. **193:**413–421.
7. **Bofill-Mas, S., N. Albinana-Gimenez, P. Clemente-Casares, A. Hundesa, J. Rodriguez-Manzano, A. Allard, M. Calvo, and R. Girones.** 2006. Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. Appl. Environ. Microbiol. **72:**7894–7896.
8. **Bosch, A.** 1998. Human enteric viruses in the water environment: a minireview. Int. Microbiol. **3:**191–196.
9. **Bryce, J., C. Boschi-Pinto, K. Shibuya, and R. E. Black.** 2005. WHO estimates of the causes of death in children. Lancet **365:**1147–1152.
10. **Bull, R. A., E. T. Tu, C. J. McIver, W. D. Rawlinson, and P. A. White.** 2006. Emergence of a new norovirus genotype II.4 variant associated with global outbreaks of gastroenteritis. J. Clin. Microbiol. **44:**327–333.
11. **Carducci, A., M. Verani, R. Battistini, F. Pizzi, E. Rovini, E. Andreoli, and B. Casini.** 2006. Epidemiological surveillance of human enteric viruses by monitoring of different environmental matrices. Water Sci. Technol. **54:**239–244.
12. **Casey, J. F., J. R. Kahn, and A. Rivas.** 2006. Willingness to pay for improved water service in Manaus, Amazonas, Brazil. Ecol. Econ. **58:**365–372.
13. **Castignolles, N., F. Petit, I. Mendel, L. Simon, L. Cattolico, and C. Buffet-Janvresse.** 1998. Detection of adenovirus in the waters of the Seine River estuary by nested-PCR. Mol. Cell. Probes **12:**175–180.
14. **Castilho, J. G., V. Munford, H. R. Resque, U. Fagundes-Neto, J. Vinjé, and M. L. Racz.** 2006. Genetic diversity of norovirus among children with gastroenteritis in Sao Paulo State, Brazil. J. Clin. Microbiol. **44:**3947–3953.
15. **Chapron, C. D., N. A. Ballester, J. H. Fontaine, C. N. Frades, and A. B. Margolin.** 2000. Detection of astroviruses, enteroviruses, and adenovirus types 40 and 41 in surface waters collected and evaluated by the information collection rule and an integrated cell culture-nested PCR procedure. Appl. Environ. Microbiol. **66:**2520–2525.
16. **Cho, H. B., S. H. Lee, J. C. Cho, and S. J. Kim.** 2000. Detection of adenoviruses and enteroviruses in tap water and river water by reverse transcription multiplex PCR. Can. J. Microbiol. **46:**417–424.
17. **Clesceri, L. S., A. E. Greenberg, and A. D. Eaton (ed.).** 1998. Standard methods for the examination of water and wastewater, 20th edition, p. 906. American Public Health Association, Washington, DC.
18. **Couceiro, S. R. M., N. Hamada, S. L. B. Luz, B. R. Forsberg, and T. P. Pimentel.** 2007. Deforestation and sewage effects on aquatic macroinvertebrates in urban streams in Manaus, Amazonas, Brazil. Hydrobiology **575:**271–284.
19. **Cunliffe, N. A., W. Dove, J. S. Gondwe, B. D. Thindwa, J. Greensill, J. L. Holmes, J. S. Bresee, S. S. Monroe, R. I. Glass, R. L. Broadhead, M. E. Molyneux, and C. A. Hart.** 2002. Detection and characterization of human astroviruses in children with acute gastroenteritis in Blantyre, Malawi. J. Med. Virol. **67:**563–566.
20. **Das, B. K., J. R. Gentsch, H. G. Cicirello, P. A. Woods, A. Gupta, M. Ramachandran, R. Kumar, M. K. Bhan, and R. I. Glass.** 1994. Characterization of rotavirus strains from newborns in New Delhi, India. J. Clin. Microbiol. **32:**1820–1822.
21. **De Paula, V. S., L. Diniz-Mendes, L. M. Villar, S. B. Luz, M. S. Jesus, N. M. V. S. da Silva, and A. M. C. Gaspar.** 2007. Hepatitis A virus in environmental water samples from the Amazon basin. Water Res. **41:**1169–1176.
22. **Divizia, M., R. Gabrieli, D. Donia, A. Macaluso, A. Bosch, S. Guix, G. Sánchez, C. Villena, R. M. Pintó, L. Palombi, E. Buonuomo, F. Cenko, L. Leno, D. Bebeci, and S. Bino.** 2004. Waterborne gastroenteritis outbreak in Albania. Water Sci. Technol. **50:**57–61.
23. **Espul, C., N. Martinez, J. S. Noel, H. Cuello, C. Abrile, S. Grucci, R. Glass, T. Berke, and D. O. Matson.** 2004. Prevalence and characterization of astroviruses in Argentinean children with acute gastroenteritis. J. Med. Virol. **72:**75–82.
24. **Fankhauser, R. L., S. S. Monroe, J. S. Noel, C. D. Humphrey, J. S. Bresee, U. D. Parashar, T. Ando, and R. I. Glass.** 2002. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. J. Infect. Dis. **186:**1–7.
25. **Galdiero, E., A. Marinelli, M. G. Pisciotta, I. Pagliara, E. S. Di Monteforte, and G. Liguori.** 2005. Reverse transcriptase-PCR for the detection of astrovirus in children with nosocomial acute diarrhoea in Naples, Italy. Med. Mal. Infect. **35:**213–217.
26. **Gallay, A., H. De Valk, M. Cournot, B. Ladeuil, C. Hemery, C. Castor, F. Bon, F. Megraud, P. Le Cann, and J. C. Desenclos.** 2006. A large multipathogen waterborne community outbreak linked to faecal contamination of a groundwater system, France, 2000. Clin. Microbiol. Infect. **12:**561–570.
27. **Gallimore, C. I., M. A. Barreiros, D. W. Brown, J. P. Nascimento, and J. P. G. Leite.** 2004. Noroviruses associated with acute gastroenteritis in a children's day care facility in Rio de Janeiro, Brazil. Braz. J. Med. Biol. Res. **37:**321–326.
28. **Gentsch, J. R., R. I. Glass, P. Woods, V. Gouvea, M. Gorziglia, J. Flores, B. K. Das, and M. K. Bhan.** 1992. Identification of group A rotavirus gene 4 types by polymerase chain reaction. J. Clin. Microbiol. **30:**1365–1373.
29. **Gofti-Laroche, L., B. Gratacap-Cavallier, D. Demanse, O. Genoulaz, J. M. Seigneurin, and D. Zmirou.** 2003. Are waterborne astrovirus implicated in acute digestive morbidity (E.MI.R.A. study)? J. Clin. Virol. **27:**74–82.
30. **Gutiérrez, M. F., M. V. Alvarado, E. Martinez, and N. J. Ajami.** 2007. Presence of viral proteins in drinkable water—sufficient condition to consider water a vector of viral transmission? Water Res. **41:**373–378.
31. **Hoebe, C. J., H. Vennema, A. M. de Roda Husman, and Y. T. van Duynhoven.** 2004. Norovirus outbreak among primary schoolchildren who had played in a recreational water fountain. J. Infect. Dis. **189:**699–705.
32. **Ijzerman, M. M., D. R. Dahling, and G. S. Fout.** 1997. A method to remove environmental inhibitors prior to the detection of waterborne enteric viruses by reverse transcription-polymerase chain reaction. J. Virol. Methods **63:**145–153.
33. **Ike, A. C., S. O. Brockmann, K. Hartelt, R. E. Marschang, M. Contzen, and R. M. Oehme.** 2006. Molecular epidemiology of norovirus in outbreaks of gastroenteritis in southwest Germany from 2001 to 2004. J. Clin. Microbiol. **44:**1262–1267.
34. **Kapikian, A. Z., and R. M. Chanock.** 2001. Rotaviruses, p. 1657–1708. *In* B. N. Fields, D. M. Knipe, and P.M. Howley (ed.), Virology, vol. 2. Lippincott-Raven Press, Philadelphia, PA.
35. **Katayama, H., A. Shimasaki, and S. Ohgaki.** 2002. Development of a virus concentration method and its application to detection of enterovirus and Norwalk virus from coastal seawater. Appl. Environ. Microbiol. **68:**1033–1039.
36. **Kim, S. H., D. S. Cheon, J. H. Kim, D. H. Lee, W. H. Jheong, Y. J. Heo, H. M. Chung, Y. Jee, and J. S. Lee.** 2005. Outbreaks of gastroenteritis that occurred during school excursions in Korea were associated with several waterborne strains of norovirus. J. Clin. Microbiol. **43:**4836–4839.
37. **Kosek, M., C. Bern, and R. L. Guerrant.** 2003. The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. Bull. W.H.O. **81:**197–201.
38. **Kukkula, M., P. Arstila, M. L. Klossner, L. Maunula, C. H. Bonsdorff, and P. Jaatinen.** 1997. Waterborne outbreak of viral gastroenteritis. Scand. J. Infect. Dis. **29:**415–418.
39. **Magalhães, F., and E. Rojas.** 2005. The re-urbanization of the city center of Manaus, Brazil—facing the challenges of informal settlements. 41st ISOCaRP Congress.
40. **Meleg, E., F. Jakab, B. Kocsis, K. Bányai, B. Melegh, and G. Szucs.** 2006. Human astroviruses in raw sewage samples in Hungary. J. Appl. Microbiol. **101:**1123–1129.
41. **Melo, E. G. F., M. S. R. Silva, and S. A. F. Miranda.** 2005. Antropic influence on the water of streams in the city of Manaus. Caminhos Geogr. **5:**40–47.
42. **Metcalf, T. G., and, X. Jiang.** 1988. Detection of hepatitis A virus in estuarine samples by gene probe assay. Microbiol. Sci. **5:**296–300.

43. **Noel, J., T. W. Lee, J. B. Kurtz, R. I Glass, and S. S. Monroe.** 1995. Typing of human astroviruses from clinical isolates by enzyme immunoassay and nucleotide sequencing. J. Clin. Microbiol. **33:**797–801.
44. **Parashar, U. D., C. J. Gibson, J. S. Bresse, and R. I. Glass.** 2006. Rotavirus and severe childhood. Emerg. Infect. Dis. **12:**304–306.
45. **Payment, P., and P. H. Hunter.** 2001. Endemic and epidemic infectious intestinal disease and its relationship to drinking water, p. 61–88. *In* L. Fewtrell and J. Bartram (ed.), Water quality: guidelines, standards and health. Assessment of risk and risk management for water-related infectious disease. IWA Publishing, London, United Kingdom.
46. **Puig, M., J. Jofre, F. Lucena, A. Allard, G. Wadell, and R. Girones.** 1994. Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. Appl. Environ. Microbiol. **60:**2963–2970.
47. **Pusch, D., D.-Y. Oh, S. Wolf, R. Dumke, U. Schroter-Bobsin, M. Hohne, I. Roske, and E. Schreier.** 2005. Detection of enteric viruses and bacterial indicators in German environmental waters. Arch. Virol. **150:**929–947.
48. **Rose, M. A., A. K. Dhar, H. A. Brooks, F. Zecchini, and R. M. Gersberg.** 2006. Quantification of hepatitis A virus and enterovirus levels in the lagoon canals and Lido beach of Venice, Italy using real time RT-PCR. Water Res. **40:**2378–2396.
49. **Santos, N., and Y. Hoshino.** 2005. Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. Rev. Med. Virol. **15:**29–56.
50. **Shieh, Y. S., D. Wait, L. Tai, and M. D. Sobsey.** 1995. Methods to remove inhibitors in sewage and other fecal wastes for enterovirus detection by the polymerase chain reaction. J. Virol. Methods **54:**51–66.
51. **Silva, P. A., D. D. Cardoso, and E. Schreier.** 2006. Molecular characterization of human astroviruses isolated in Brazil, including the complete sequences of astrovirus genotypes 4 and 5. Arch. Virol. **151:**1405–1417.
52. **Standish-Lee, P., and E. Loboschefsky.** 2006. Protecting public health from the impact of body-contact recreation. Water Sci. Technol. **53:**201–207.
53. **Straub, T. M., and D. P. Chandler.** 2003. Towards a unified system for detecting waterborne pathogens. J. Microbiol. Methods **53:**185–197.
54. **Villar, L. M., V. S. de Paula, L. Diniz-Mendes, E. Lampe, and A. M. Gaspar.** 2006. Evaluation of methods used to concentrate and detect hepatitis A virus in water samples. J. Virol. Methods **137:**169–179.
55. **Wilhelmi, I., E. Roman, and A. Sánchez-Fauquier.** 2003. Viruses causing gastroenteritis. Clin. Microbiol. Infect. **9:**247–262.
56. **Wyn-Jones, A. P., and J. Sellwood.** 2001. Enteric viruses in the aquatic environment. J. Appl. Microbiol. **91:**945–962.

5. DISCUSSÃO

5.1. Diversidade genética do torque teno virus (artigo 1)

O estudo da variabilidade auxilia a identificar novos genótipos ou grupos filogenéticos envolvidos com o desenvolvimento de patogenias e contribui para um melhor entendimento sobre a taxonomia do TTV. Desta forma, a investigação da variabilidade genética requer o emprego de análise do genoma completo. Com a utilização da RCA, é possível amplificar moléculas de DNA circular (e.g., genomas circulares, plasmídeos) com grande eficiência (Dean et al. 2001) e fidelidade (Garmendia et al. 1992). Este protocolo apresenta duas vantagens: (i) a utilização da enzima polimerase de alta fidelidade e estabilidade do bacteriófago phi29 e, (ii) o emprego de oligonucleotídeos iniciadores randômicos que viabilizam a amplificação de seqüências sem a necessidade do conhecimento prévio da seqüência-alvo. Utilizando-se este sistema, dois genomas completos de TTVs humanos, originados de um único paciente e, dois genomas de TTVs suínos, originados de um único porco, puderam ser amplificados, seqüenciados e caracterizados (artigo 1). Este trabalho foi o primeiro no mundo a empregar esta técnica a fluidos corporais. Os dois genomas amplificados a partir de soros humanos puderam ser classificados no genogrupo 3 do TTV. Contudo, os dois clones de TTV de suínos (1p e 2p) apresentaram um grau de identidade muito baixo entre si (43%) e em comparação com a seqüência de nucleotídeos de genoma completo disponível no Genbank no momento (45%). De acordo com os critérios utilizados para a classificação de TTV humanos, o clone 2p (Sd-TTV2p) pôde ser considerado o protótipo de um novo grupo genômico de TTV suíno, classificado como genogrupo 2.

5.2. Torque teno virus como indicador de contaminação de natureza antrópica em ecossistemas aquáticos

Em 2003, antendendo ao edital temático "Qualidade de Vida e Gestão de Recursos Hídricos: Água - Caminhos para garantir a vida" do Programa Institucional de Pesquisa em Saúde & Ambiente da Vice-Presidência de Serviços de Referência e Ambiente da FIOCRUZ, os laboratórios de

Virologia Molecular (TTV), Desenvolvimento Tecnológico (HAV) e Virologia Comparada (AdV, RV, NoV e AstV) iniciaram projeto de pesquisa de vírus entéricos no ambiente. Neste momento, o Laboratório de Desenvolvimento Tecnológico estava desenvolvendo pesquisas na linha de Virologia Ambiental para estabelecimento de metodologia para concentração de partículas virais a partir de diferentes amostras de água.

5.2.1. Estabelecimento de método de concentração viral (artigo 2)

Como citado anteriormente, o volume de água pode constituir um fator limitante para a pesquisa de vírus. Desta forma, um método capaz de recuperar vírus a partir de pequenos volumes torna esta prática mais acessível, viabilizando um maior alcance dentre os métodos de monitoramento ambiental.

Neste estudo, dois métodos capazes de concentrar vírus a partir de pequenos volumes de água (2 L e 0,5 L) foram avaliados, utilizando a cepa HAF-203 de HAV como modelo, em estudos experimentais pela inoculação em diferentes amostras de água. Os protocolos baseados na adsorção de partículas virais a membranas de carga negativa na presença de íons magnésio apresentavam diferença na etapa de eluição e de reconcentração. No protocolo 1 (Jothikumar et al. 1993), as partículas foram concentradas a partir de uma coleta de 0,5 L de amostra inicial e a eluição ocorreu em tampão urea-arginina fosfato e a reconcentração se deu pela centrifugação após floculação das partículas na presença de $MgCl_2$. O método 2 (Katayama et al. 2002) foi modificado pela adição de $MgCl_2$ aos 2 L de amostra inicial até a concentração final de 3 M, que atuou como agente facilitador da adsorção da partícula viral à membrana eletronegativa. Ainda neste protocolo, as partículas foram eluídas em NaOH e, após neutralização, reconcentradas por ultrafiltração utilizando o Centriprep® YM-50 (Milipore).

As duas metodologias empregadas foram capazes de concentrar as partículas de HAV com taxas de recuperação de cerca de 90%. Pelo PCR quantitativo, o RNA viral pôde ser detectado nos quatro tipos diferentes de

água avaliados (rio, mar, torneira e mineral). Contudo, o PCR qualitativo não foi capaz de detectar a presença do RNA do HAV nas amostras de água costeira, provavelmente devido a presença de inibidores da polimerase e, a maior susceptibilidade do PCR qualitativo a estes tipos de substâncias. A análise qualitativa e quantitativa de detecção demonstrou que o método 1 foi mais apropriado para monitoramento de HAV em água de torneira enquanto que o método 2 foi mais adequado no estudo das águas mineral e de rio. Este fato pode refletir a necessidade de se empregar diferentes metodologias para cada tipo de água.

Estabelecida a metodologia de concentração para detecção de HAV em água de rio, esta foi empregada para a pesquisa de vírus entéricos no projeto intitulado "Impacto da qualidade da água de Igarapés da Amazônia Central na Saúde da População" aprovado pelo Edital Água é Vida da FIOCRUZ.

5.2.2. Detecção, quantificação e caracterização molecular de torque teno virus na bacia Amazônica (artigo 3)

Em colaboração com o Laboratório de Biodiversidade do Centro de Pesquisas Leônidas & Maria Deane (FIOCRUZ-AM) e com o Instituto Nacional de Pesquisas da Amazônia (INPA) foram realizadas quatro campanhas trimestrais de coleta de água no período de agosto de 2004 a junho de 2005 em igarapés da cidade de Manaus (AM). Treze pontos de coleta foram definidos na rede hidrográfica formada pelas bacias do Tarumã-Açu, Mindu-São Raimundo e Quarenta-Educandos, onde está localizado o município de Manaus, com 1.403.796 habitantes. Esses pontos foram precisamente localizados utilizando-se o Sistema de Posicionamento Global – GPS (TREX Legend – Gramin Ltda – Olathe, Kansas, USA). A Figura 7 apresenta as diferentes bacias e sua localização na cidade de Manaus.

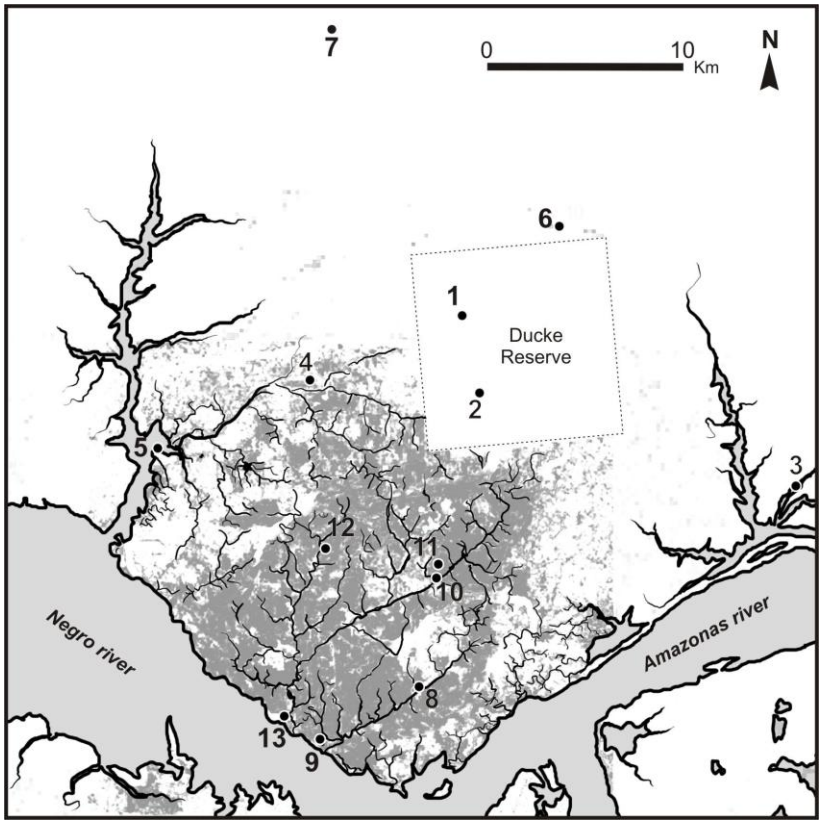

Figura 7. Representação da imagem de satélite das microbacias hidrográficas de Manaus-AM. Os números de 1 a 13 indicam os pontos onde foram realizadas as coletas de amostras de água. A região do mapa em cinza indica a área urbana.

Os pontos de coleta de amostras de águas superficiais foram definidos de acordo com diferentes áreas de ocupação humana seguindo os seguintes critérios: a) três pontos controle de Floresta primária, localizados em área de reserva florestal, com vegetação intacta, acesso humano restrito e livre de esgoto doméstico; b) dois pontos em uma área descampada aonde a ocupação humana é reduzida e apresentam poucos pontos de despejo de esgoto doméstico e c) oito pontos localizados em áreas com diferentes graus de influência antrópica sem serviço de tratamento de água ou esgoto.

A aplicação da técnica de qPCR, no sistema TaqMan, para detecção e quantificação da carga viral de TTV nas amostras de água foi avaliada neste estudo. Existem muitas vantagens na aplicação deste tipo de ensaio: (i) é um método rápido, a detecção é realizada em aproximadamente 2 h, (ii) apresenta um risco menor de contaminação cruzada que poderia gerar

resultados falso-positivos e, (iii) apresenta especificidade maior ou igual a outras formas de detecção como a PCR 'nested', devido à hibridização de sonda especifica no produto de PCR. A possibilidade de quantificar os vírus presentes nas amostras é o ponto mais importante neste procedimento. O número de cópias genoma viral é estimado pela comparação a uma curva padrão após um determinado número de ciclos de amplificação na mesma reação (Jothikumar et al. 2005). A aplicabilidade da qPCR em tempo real para detecção de vírus de forma rápida em vários tipos de amostras ambientais representa um avanço considerável e um grande potencial para aplicações no estudo do meio ambiente (He & Jiang, 2005). A metodologia de qPCR desenvolvida neste estudo apresentou sensibilidade para detecção de 500 genomas-equivalente (gEq)/100 mL de água de rio (artigo 3). O TTV foi detectado nas amostras provenientes de Manaus em concentrações que variaram de 10^3 a 10^5 gEq/100 mL, demonstrando sua grande disseminação neste ambiente. Este método foi implantado no laboratório a partir deste trabalho e vem sendo desde então empregado em outras linhas de pesquisa sobre este vírus.

A diversidade genética do TTV foi observada nas amostras ambientais estudadas, sendo detectado por PCR, utilizando oligonucleotídeos iniciadores capazes de amplificar a maioria, senão todos os genótipos conhecidos de TTV humano (Takahashi et al. 1998). A análise filogenética das seqüências de nucleotídeos dos produtos de PCR obtidos identificou a presença de 11 seqüências distintas entre um total de 19 amplificados. Apesar da elevada sensibilidade do ensaio de PCR utilizado, o produto gerado é pequeno (199 bp), não sendo possível a determinação do genótipo e/ou grupo genômico destes vírus. A obtenção de seqüências nucleotídicas destes produtos foi empregada para a confirmação da origem humana e da avaliação da diversidade genética nas amostras.

Despejos de esgoto sem tratamento são responsáveis pela presença destes vírus no leito de rios e lagos e a análise da sua composição constitui uma forma de se avaliar a diversidade de vírus circulantes na população de uma determinada área geográfica (artigo 3).

5.2.3. Torque teno virus como marcador de poluição antrópica (artigos 3, 4 e 5)

O método de concentração estabelecido foi empregado com sucesso na detecção de genomas de outros vírus entéricos. Além do TTV (artigo 3) e do HAV (artigo 4), os AdV, RV, NoV e AstV (artigo 5) foram pesquisados. Os vírus mais prevalentes dentre os avaliados por PCR convencional foram o RV, com 44 % de prevalência, seguido do TTV (35 %), AdV (31 %), HAV (23%), AstV(15 %) e NoV (6 %) (tabela 1).

Além do TTV (artigo 3) o HAV (artigo 4) também foi detectado e quantificado por PCR em tempo real. A utilização desta metodologia elevou o percentual de amostras positivas de ambos os vírus para 92% demonstrando a importância do método de detecção viral para o monitoramento destes vírus. O genoma do HAV pôde ser detectado em concentrações que variaram de 10^0 a 10^2 gEq/100 mL de amostra coletada, sendo mil vezes menor que as concentrações observadas para o TTV.

A presença o TTV não se mostrou relacionada à presença de nenhum dos outros vírus entéricos avaliados nos igarapés de Manaus. Na mesma área, o AdV foi detectado por PCR *nested* em 31% das amostras (artigo 5), sendo 13 delas detectadas em áreas de alto impacto antrópico. Embora o AdV tenha sido apontado como um marcador de contaminação ambiental (Jiang, 2006), neste estudo o TTV apresentou uma prevalência superior sugerindo sua utilização nesta finalidade. Neste estudo a carga viral do AdV não foi avaliada sendo necessário novos estudos comparativos para melhor avaliação destes vírus como marcadores de contaminação ambiental. A alta prevalência de RV, superior aos do vírus de DNA, pode ser explicada pela endemicidade deste vírus na região. Os resultados obtidos sobre a contaminação viral nas microbacias da região amazônica demonstram a vulnerabilidade deste ecossistema aquático e evidenciam o impacto do processo de ocupação desordenada ocorrida nas últimas décadas. (Projeto Geocidades, 2002).

Tabela 1. Prevalência dos vírus entéricos avaliados nos igarapés urbanos na bacia Amazônica, Manaus, Amazonas.

Ponto	Área	Mês de Coleta			
		Agosto 2004	Novembro 2004	Fevereiro 2005	Junho 2005
1	Floresta	RV, TTV	-	RV	-
2		TTV	TTV	-	AdV, HAV, TTV
3		TTV	RV, TTV	-	-
4	Rural	RV	TTV	-	RV
5		AstV	AstV, RV, HAV	RV, TTV	HAV
6		TTV	-	TTV	RV, TTV
7		TTV	-	-	TTV
8	Urbana	AdV, RV, HAV	AdV	AdV, RV	RV, TTV
9		AdV, HAV	AdV, HAV	NoV, RV	AdV, RV
10		AstV, AdV, HAV, TTV	AdV, HastV, NoV, HAV, TTV	AdV, AstV, RV, NoV	AdV, AstV, RV, TTV
11		HAV	AdV, RV	AdV, AstV, RV, HAV	AdV, HAV
12		TTV	RV, TTV	RV	-
13		AdV, RV	RV	RV	AdV, AstV, RV

Legenda: TTV (torque teno vírus), HAV (vírus da hepatite A), AdV (adenovírus humanos), RV (rotavírus), NoV (norovírus) e AstV (astrovírus humanos)

A comparação das prevalências do TTV com as prevalências dos demais vírus entéricos, obtidas pela PCR convencional não apresentou nenhuma correlação estatisticamente significativa com a presença de nenhum dos outros vírus entéricos avaliados, nem com a presença de coliformes fecais acima dos níveis permitidos pela legislação (Tabela 2).

Tabela 2. Comparação da prevalência do torque teno virus obtida pela PCR qualitativo com os outros vírus entéricos avaliados e com coliformes fecais.

Vírus	Áreas					
	Floresta (*n*=12)		Rural (*n*=16)		Urbana (*n*=24)	
	x^2	*P*	x^2	*P*	x^2	*P*
HAdV	1,477	0,224	*b*	*b*	0,523	0,470
HAV	1,477	0,224	2,522	0,112	0,000	1,000
RV	0,451	0,502	0,042	0,838	0,523	0,470
NoV	*b*	*b*	*b*	*b*	0,120	0,729
HAstV	*b*	*b*	2,522	0,112	2,454	0,117
Qualquer vírus	0,000	1,000	0,429	0,513	0,637	0,425
Colif. Fecais[a]	0,345	0,557	0,912	0,340	*c*	*c*

a = considerados positivos todos os que excederam a quantidade de 1000 CFU/100 mL.
b = os valores de qui-quadrado e de *P* não puderam ser calculados porque a variável 'vírus' é constante (todos negativos).
c = os valores de qui-quadrado e de *P* não puderam ser calculados porque a variável 'coliformes fecais' é constante (todos positivos).

Deve-se levar ainda em consideração que condições sub-ótimas de detecção, resultantes da ação de inibidores da polimerase, presentes em amostras ambientais, podem alterar o perfil de prevalência e quantificação (Fong nd Lipp, 2005). O fato de quatro amostras inicialmente negativas terem se tornado positivas pela qPCR, após diluição de 10 vezes, constitui um forte indício para a presença de inibidores. A presença do ácido húmico, resultante da decomposição de matéria orgânica, presente na região amazônica (Goulding et al. 1988), pode ser responsável por uma subestimação na detecção/quantificação das amostras provenientes da área de estudo.

A alta prevalência de TTV encontrada revela a grande disseminação deste vírus na área estudada, inclusive nas regiões de reserva próximas a nascentes, pouco impactadas, onde os parâmetros físico-químicos e

bacteriológicos estavam dentro dos padrões de balneabilidade. Este resultado demonstra que a presença de TTV é uma indicação sensível de contaminação fecal humana. Neste trabalho não foi observada nenhuma correlação direta entre o nível de poluição, determinado por medição de parâmetros físico-químicos e bacteriológicos, e a carga viral do TTV (artigo 3).

A presença do TTV não apresentou correlação significativa com a estação do ano ou intensidade pluviométrica. A relação de infecção crônica que o TTV costuma manter com espécie humana se reflete na sua excreção independente de fatores sazonais. Por outro lado, a elevada prevalência, inclusive em indivíduos saudáveis, torna sua presença independente do aparecimento de surtos. Outra característica importante é dada pela especificidade do ensaio de PCR para a detecção de TTVs de origem humana, não havendo risco de detecção/quantificação cruzada com os TTVs de animais corroborando seu potencial como indicador antrópico de poluição.

6. CONCLUSÕES

1. As sequências de TTV analisados neste estudo apresentaram grande diversidade nucleotídica, sendo possível a descrição de dois novos genótipos de TTV humano e de um novo grupo genômico de TTV suíno denominado Sd-TTV2p que passa a ser o protótipo do genogrupo 2 de TTV suíno.

2. O protocolo de concentração de partículas virais utilizando membrana carregada negativamente, modificado pela adição de íons magnésio, e com reconcentrção por ultrafiltração se mostrou eficaz para recuperação de HAV em amostras de rio.

3. O protocolo de concentração de partículas virais estabelecido foi empregado em amostras de água de igarapés da bacia Amazônica e se mostrou eficaz para o monitoramento da presença dos vírus analisados neste estudo (TTV, HAV, AdV, RV, NoV, HAstV).

4. A utilização da qPCR para a detecção de HAV no ambiente elevou o percentual de amostras positivas de 23% para 92% demonstrando a importância do método de detecção viral para o monitoramento destes vírus. O genoma do HAV pôde ser detectado em concentrações que variaram de 10^0 a 10^2 gEq/100 mL de amostra coletada.

5. A utilização da qPCR para a detecção de TTV no ambiente elevou o percentual de amostras positivas de 37% para 92% confirmando a importância do método quantitativo de detecção para o monitoramento de vírus em amostras ambientais. O genoma do TTV pôde ser detectado em concentrações que variaram de 10^3 a 10^5 gEq/100 mL de amostra coletada demonstrando a elevada concentração deste vírus nas amostras estudadas.

6. A PCR qualitativa, embora menos sensível que a qPCR, foi importante para a avaliação da diversidade genética do TTV e confirmação da origem humana por sequenciamento nucleotídico do produto obtido.

7. A grande diversidade do TTV nas amostras ambientais foi demonstrada pela grande variabilidade das seqüências nucleotídicas obtidas. Dentre as 19

amostras que puderam ser amplificadas, 11 seqüências distintas puderam ser observadas.

8. O resultado de detecção qualitativa dos vírus entéricos nas amostras de água dos igarapés de Manaus revelou maior prevalência de RV (44%) quando comparada a de TTV (37%), que pode ser explicada pela endemicidade deste patógeno na área estudada.

9. A utilização da qPCR revelou a mesma prevalência de TTV e HAV sendo a concentração de TTV (10^5) no ambiente mil vezes superior a de HAV (10^2).

10. A análise global dos dados obtidos neste estudo demonstra o potencial do TTV humano como indicador de poluição antrópica, embora novos estudos devam ser realizados para a comprovação do papel que o TTV pode assumir para tornar-se um parâmetro de avaliação de qualidade da água.

7. PERSPECTIVAS

O estabelecimento da metodologia de concentração, detecção e quantificação do TTV em amostras ambientais abre novas perspectivas para o estudo deste vírus em diferentes matrizes. Diante dos fortes indícios levantados neste estudo quanto à possibilidade do uso do TTV como indicador de poluição de natureza antrópica, a metodologia estabelecida no presente trabalho será aplicada a amostras de esgoto antes e após o tratamento. Os resultados de detecção do TTV serão comparados aos de outros patógenos considerados atualmente como referência neste sentido como os adenovírus e os coliformes.

8. REFERÊNCIAS BIBLIOGRÁFICAS

Abbaszadegan M, Stewart P, LeChevallier M. A strategy for detection of viruses in groundwater by PCR. Appl Environ Microbiol. 1999 Feb;65(2):444-9.

Abe K, Inami T, Asano K, Miyoshi C, Masaki N, Hayashi S, Ishikawa K, Takebe Y, Win KM, El-Zayadi AR, Han KH, Zhang DY. TT virus infection is widespread in the general populations from different geographic regions. J Clin Microbiol. 1999 Aug;37(8):2703-5.

Appleton H. Control of food-borne viruses. Br Med Bull. 2000;56(1):172-83.

Asabe S, Nishizawa T, Iwanari H, Okamoto H. Phosphorylation of serine-rich protein encoded by open reading frame 3 of the TT virus genome. Biochem Biophys Res Commun. 2001 Aug 17;286(2):298-304.

Bendinelli M, Pistello M, Maggi F, Fornai C, Freer G, Vatteroni ML. Molecular properties, biology, and clinical implications of TT virus, a recently identified widespread infectious agent of humans. Clin Microbiol Rev. 2001 Jan;14(1):98-113.

Berns, KI. Parvoviridae: the virus and their replication. In: FIELDS, B. N. *et al*. *Fields virology*. 3 ed. Philadelphia: Lippincot-Raven, 1996. p. 2173-99.

Biagini P, Gallian P, Attoui H, Cantaloube JF, de Micco P, de Lamballerie X. Determination and phylogenetic analysis of partial sequences from TT virus isolates. J Gen Virol. 1999 Feb;80(Pt 2):419-24.

Biagini P, Gallian P, Attoui H, Cantaloube JF, Touinssi M, de Micco P, de Lamballerie X. Comparison of systems performance for TT virus detection using PCR primer sets located in non-coding and coding regions of the viral genome. J Clin Virol. 2001 Aug;22(1):91-9.

Biagini P, Gallian P, Cantaloube JF, Attoui H, de Micco P, de Lamballerie X. Distribution and genetic analysis of TTV and TTMV major phylogenetic groups in French blood donors. J Med Virol. 2006 Feb;78(2):298-304.

Biagini P, Todd D, Bendinelli M, Hino S, Mankertz A, Mishiro S, Niel C, Okamoto H, Raidal S, Ritchie BW, Teo GC. Anellovirus in virus taxonomy. In Eight report of the international committee on taxonomy of viruses. Eds. Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA. London, United Kingdom: Elsevier/Academic Press. p. 335-341, 2004.

Biagini P, Uch R, Belhouchet M, Attoui H, Cantaloube JF, Brisbarre N, de Micco P. Circular genomes related to anelloviruses identified in human and animal samples by using a combined rolling-circle amplification/sequence-independent single primer amplification approach. J Gen Virol. 2007 Oct;88(Pt 10):2696-701.

Borchardt MA, Bertz PD, Spencer SK, Battigelli DA. Incidence of enteric viruses in groundwater from household wells in Wisconsin. Appl Environ Microbiol. 2003 Feb;69(2):1172-80.

Bosch A. Human enteric viruses in the water environment: a minireview. Int Microbiol. 1998 Sep;1(3):191-6.

Calcaterra S, Zaniratti MS, Serraino D, Peroni M, Abbate I, Cappiello G, Piselli P, Pavia C, Rezza G, Ippolito G, Capobianchi MR. Cervicovaginal shedding of TT virus in HIV-infected women. J Hum Virol. 2001 Nov-Dec;4(6):343-5.

Carducci A, Casini B, Bani A, Rovini E, Verani M, Mazzoni F, Giuntini A. Virological control of groundwater quality using biomolecular tests. Water Sci Technol. 2003;47(3):261-6.

Chen BP, Rumi MG, Colombo M, Lin YH, Ramaswamy L, Luna J, Liu JK, Prati D, Mannucci PM. TT virus is present in a high frequency of Italian hemophilic patients transfused with plasma-derived clotting factor concentrates.Blood. 1999 Dec 15;94(12):4333-6.

Dean FB, Nelson JR, Giesler TL, Lasken RS. Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. Genome Res. 2001 Jun;11(6):1095-9.

Deng X, Terunuma H, Handema R, Sakamoto M, Kitamura T, Ito M, Akahane Y. Higher prevalence and viral load of TT virus in saliva than in the corresponding serum: another possible transmission route and replication site of TT virus. J Med Virol. 2000 Dec;62(4):531-7.

Devalle S, Niel C. Distribution of TT virus genomic groups 1-5 in Brazilian blood donors, HBV carriers, and HIV-1-infected patients. J Med Virol. 2004 Jan;72(1):166-73.

Erensoy S, Sayiner AA, Turkoglu S, Canatan D, Akarca US, Sertoz R, Ozacar T, Batur Y, Badur S, Bilgic A. TT virus infection and genotype distribution in blood donors and a group of patients from Turkey. Infection 2002 Oct;30(5):299-302.

Erker JC, Leary TP, Desai SM, Chalmers ML, Mushahwar IK. Analyses of TT virus full-length genomic sequences. J.Gen.Virol. 1999 Jul;80(Pt 7):1743-50.

Fong TT, Lipp EK. Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. Microbiol Mol Biol Rev. 2005 Jun;69(2):357-71.

Frost FJ, Kunde TR, Craun GF. Is contaminated groundwater an important cause of viral gastroenteritis in the United States? J Environ Health. 2002 Oct;65(3):9-14, 38

Garmendia C, Bernad A, Esteban JA, Blanco L, Salas M. The bacteriophage phi 29 DNA polymerase, a proofreading enzyme. J Biol Chem. 1992 Feb 5;267(4):2594-9.

Gersberg RM, Rose MA, Robles-Sikisaka R, Dhar AK. Quantitative detection of hepatitis a virus and enteroviruses near the United States-Mexico border and correlation with levels of fecal indicator bacteria. Appl Environ Microbiol. 2006 Dec;72(12):7438-44.

Gibbs, MJ, and Weiller GF. 1999. Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. Proc Natl Acad Sci. USA 96:8022–27.

Gilgen M, Germann D, Lüthy J, Hübner P. Three-step isolation method for sensitive detection of enterovirus, rotavirus, hepatitis A virus, and small round structured viruses in water samples. Int J Food Microbiol. 1997 Jul 22;37(2-3):189-99.

Gofti-Laroche L, Gratacap-Cavallier B, Genoulaz O, Joret JC, Hartemann P, Seigneurin JM, Zmirou D. A new analytical tool to assess health risks associated with the virological quality of drinking water (EMIRA study). Water Sci Technol. 2001;43(12):39-48.

Goulding M, Carvalho ML & Ferreira EG. Rio Negro, rich life in poor water: Amazonian diversity and foodchain ecology as seen through fish communities. The Hague, Netherlands: SPB Academic Pub. 1988.

Griffin DW, Gibson CJ 3rd, Lipp EK, Riley K, Paul JH 3rd, Rose JB. Detection of viral pathogens by reverse transcriptase PCR and of microbial indicators by standard Methods in the canals of the Florida Keys. Appl Environ Microbiol. 1999 Sep;65(9):4118-25.

Hallett RL, Clewley JP, Bobet F, McKiernan PJ, Teo CG. Characterization of a highly divergent TT virus genome. J Gen Virol. 2000 Sep;81:2273-9.

Haramoto E, Katayama H, Oguma K, Ohgaki S. Application of cation-coated filter method to detection of noroviruses, enteroviruses, adenoviruses, and torque teno viruses in the Tamagawa River in Japan. Appl Environ Microbiol. 2005a May;71(5):2403-11.

Haramoto E, Katayama H, Oguma K, Yamashita H, Nakajima E, Ohgaki S. One-year monthly monitoring of Torque teno virus (TTV) in wastewater treatment plants in Japan. Water Res. 2005b May;39(10):2008-13.

He JW, Jiang S. Quantification of enterococci and human adenoviruses in environmental samples by real-time PCR. Appl Environ Microbiol. 2005 May;71(5):2250-5.

He Z, Zhuang H, Wang X, Song S, Dong Q, Yan J, Buehring GC, Luo G. Retrospective analysis of non-A-E hepatitis: possible role of hepatitis B and C virus infection. J Med Virol. 2003 Jan;69(1):59-65.

Hino S, Miyata H.Torque teno virus (TTV): current status. Rev Med Virol. 2007 Jan-Feb;17(1):45-57.

Hsieh SY, Wu YH, Ho YP, Tsao KC, Yeh CT, Liaw YF. High prevalence of TT virus infection in healthy children and adults and in patients with liver disease in Taiwan. J Clin Microbiol. 1999 Jun;37(6):1829-31.

Huang LY, Oystein Jonassen T, Hungnes O, Grinde B. High prevalence of TT virus-related DNA (90%) and diverse viral genotypes in Norwegian blood donors. J Med Virol. 2001 Jul;64(3):381-6.

Ijzerman MM, Dahling DR, Fout GS. A method to remove environmental inhibitors prior to the detection of waterborne enteric viruses by reverse transcription-polymerase chain reaction. J Virol Methods. 1997 Jan;63(1-2):145-53.

Inami T, Konomi N, Arakawa Y, Abe K. High prevalence of TT virus DNA in human saliva and semen. J Clin Microbiol. 2000 Jun;38(6):2407-8.

Itoh K, Takahashi M, Ukita M, Nishizawa T, Okamoto H. Influence of primers on the detection of TT virus DNA by polymerase chain reaction. J Infect Dis. 1999 Nov;180(5):1750-1.

Itoh Y, Takahashi M, Fukuda M, Shibayama T, Ishikawa T, Tsuda F, Tanaka T, Nishizawa T, Okamoto H. Visualization of TT virus particles recovered from the sera and feces of infected humans. Biochem Biophys Res Commun. 2000 Dec 20;279(2):718-24.

Jiang SC. Human adenoviruses in water: occurrence and health implications: a critical review. Environ Sci Technol. 2006 Dec 1;40(23):7132-40.

Jothikumar N, Aparna K, Kamatchiammal S, Paulmurugan R, Saravanadevi S, Khanna P. Detection of hepatitis E virus in raw and treated wastewater with the polymerase chain reaction. Appl Environ Microbiol. 1993 Aug;59(8):2558-62.

Jothikumar N, Cromeans TL, Sobsey MD, Robertson BH. Development and evaluation of a broadly reactive TaqMan assay for rapid detection of hepatitis A virus. Appl Environ Microbiol. 2005 Jun;71(6):3359-63.

Kamahora T, Hino S, Miyata H. Three spliced mRNAs of TT virus transcribed from a plasmid containing the entire genome in COS1 cells. J Virol. 2000 Nov;74(21):9980-6.´

Karaganis, JV, Larking EP, Melnick JL, Scarpino PV, Schaub SA, Sorber CA *et al*. (1983) Research priorities for monitoring viruses in the environment. In Cincinnati: U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring and Support Laboratory.

Katayama H, Shimasaki A, Ohgaki S. Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater. Appl Environ Microbiol. 2002 Mar;68(3):1033-9.

Kittigul L, Khamoun P, Sujirarat D, Utrarachkij F, Chitpirom K, Chaichantanakit N, Vathanophas K. An improved method for concentrating rotavirus from water samples. Mem Inst Oswaldo Cruz. 2001 Aug;96(6):815-21.

Kooistra K, Zhang YH, Henriquez NV, Weiss B, Mumberg D, Noteborn MH. TT virus-derived apoptosis-inducing protein induces apoptosis preferentially in hepatocellular carcinoma-derived cells. J Gen Virol. 2004 Jun;85:1445-50.

Leary TP, Erker JC, Chalmers ML, Desai SM, Mushahwar IK. Improved detection systems for TT virus reveal high prevalence in humans, non-human primates and farm animals. J Gen Virol. 1999 Aug;80(Pt 8):2115-2120.

Leclerc H, Schwartzbrod L, Dei-Cas E. Microbial agents associated with waterborne diseases. Crit Rev Microbiol. 2002;28(4):371-409.

Lee SH, Kim SJ. Detection of infectious enteroviruses and adenoviruses in tap water in urban areas in Korea. Water Res. 2002 Jan;36(1):248-56.

Li JW, Wang XW, Yuan CQ, Zheng JL, Jin M, Song N, Shi XQ, Chao FH. Detection of enteroviruses and hepatitis A virus in water by consensus primer multiplex RT-PCR. World J Gastroenterol. 2002 Aug;8(4):699-702.

Lisitsyn N, Lisitsyn N, Wigler M. Cloning the differences between two complex genomes. Science. 1993 Feb 12;259(5097):946-951.

Luo K, He H, Liu Z, Liu D, Xiao H, Jiang X, Liang W, Zhang L. Novel variants related to TT virus distributed widely in China. J Med Virol. 2002 May;67(1):118-26.

Manni F, Rotola A, Caselli E, Bertorelle G, Di Luca D. Detecting recombination in TT virus: a phylogenetic approach. J Mol Evol. 2002 Nov;55(5):563-72.

Matsubara H, Michitaka K, Horiike N, Yano M, Akbar SM, Torisu M, Onji M. Existence of TT virus DNA in extracellular body fluids from normal healthy Japanese subjects. Intervirology 2000;43(1):16-9.

Mehnert DU, Queiroz APS, Pauli V, Monezi TA, Hársi CM. Virus: a new parameter for determination of water quality. Virus Rev Res.2001;6:67.

Melnick, JL. Poliomieyelitis virus in urban sewage in epidemic and non-epidemic times. Am J Hyg 1947;45:240-53.

Metcalf TG, Melnick JL, Estes MK. Environmental virology: from detection of virus in sewage and water by isolation to identification by molecular biology--a trip of over 50 years. Annu Rev Microbiol. 1995;49:461-87.

Miyata H, Tsunoda H, Kazi A, Yamada A, Khan MA, Murakami J, Kamahora T, Shiraki K, Hino S. Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. J Virol. 1999 May;73(5):3582-6.

Mushahwar IK, Erker JC, Muerhoff AS, Leary TP, Simons JN, Birkenmeyer LG, Chalmers ML, Pilot-Matias TJ, Desai SM. Molecular and biophysical characterization of TT virus: evidence for a new virus family infecting humans. Proc Natl Acad Sci USA. 1999 Mar 16;96(6):3177-82.

Niel C, de Oliveira JM, Ross RS, Gomes SA, Roggendorf M, Viazov S. High prevalence of TT vírus infection in Brazilian blood donors. J Med Virol. 1999 Mar;57(3):259-63.

Niel C, Diniz-Mendes L, Devalle S. Rolling-circle amplification of Torque teno virus (TTV) complete genomes from human and swine sera and identification of a novel swine TTV genogroup. J Gen Virol. 2005 May;86(Pt 5):1343-7.

Ninomiya M, Takahashi M, Shimosegawa T, Okamoto H. Analysis of the entire genomes of fifteen torque teno midi virus variants classifiable into a third group of genus Anellovirus. Arch Virol. 2007b 152(11):1961-75.

Nishizawa T, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion Hepatitis of unknown etiology. Biochem Biophys Res Commun. 1997 Dec 8;241(1):92-7.

Nishizawa T, Okamoto H, Tsuda F, Aikawa T, Sugai Y, Konishi K, Akahane Y, Ukita M, Tanaka T, Miyakawa Y, Mayumi M. Quasispecies of TT virus (TTV) with sequence divergence in hypervariable regions of the capsid protein in chronic TTV infection. J Virol. 1999 Nov;73(11):9604-8.

Noble RT & Fuhrman JA. Enteroviruses detected by reverse transcriptase polimerase chain reaction from the coastal waters of Santa Monica Bay: California: low correlation to bacterial indicator levels. Hydrobiologia 2001a Set;460:175-84.

Okamoto H, Akahane Y, Ukita M, Fukuda M, Tsuda F, Miyakawa Y, Mayumi M. Fecal excretion of a nonenveloped DNA virus (TTV) associated with posttransfusion non-A-G hepatitis. J Med Virol. 1998b Oct;56(2):128-32.

Okamoto H, Fukuda M, Tawara A, Nishizawa T, Itoh Y, Hayasaka I, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. Species-specific TT viruses and cross-species infection in nonhuman primates. J Virol. 2000a Feb;74(3):1132-9.

Okamoto H, Nishizawa T, Kato N, Ukita M, Ikeda H, Iizuka H, Miyakawa Y, Mayumi M. Molecular cloning and characterization of a novel DNA virus (TTV) associated with posttransfusion hepatitis of unknown etiology. Hepatol Res. 1998a;10(1):1-16.

Okamoto H, Nishizawa T, Takahashi M, Asabe S, Tsuda F, Yoshikawa A. Heterogeneous distribution of TT virus of distinct genotypes in multiple tissues from infected humans. Virology 2001b Sep 30;288(2):358-68.

Okamoto H, Nishizawa T, Tawara A, Takahashi M, Kishimoto J, Sai T, Sugai Y. TT virus mRNAs detected in the bone marrow cells from an infected individual. Biochem Biophys Res Commun. 2000b Dec 20;279(2):700-707.

Okamoto H, Nishizawa T, Ukita M, Takahashi M, Fukuda M, Iizuka H, Miyakawa Y, Mayumi M. The entire nucleotide sequence of a TT virus isolate from the United States (TUS01): comparison with reported isolates and phylogenetic analysis. Virology. 1999d Jul 5;259(2):437-448.

Okamoto H, Nishizawa T, Ukita M. A novel unenveloped DNA virus (TT virus) associated with acute and chronic non-A to G hepatitis. Intervirology 1999c; 42(2-3):196-204.

Okamoto H, Takahashi M, Nishizawa T, Tawara A, Fukai K, Muramatsu U, Naito Y, Yoshikawa A. Genomic characterization of TT viruses (TTVs) in pigs, cats and dogs and their relatedness with species-specific TTVs in primates and tupaias. J Gen Virol. 2002 Jun;83(Pt 6):1291-7.

Okamoto H, Takahashi M, Nishizawa T, Ukita M, Fukuda M, Tsuda F, Miyakawa Y, Mayumi M. Marked genomic heterogeneity and frequent mixed infection of TT virus demonstrated by PCR with primers from coding and noncoding regions. Virology 1999b Jul 5;259(2):428-36.

Payment P, Morin E, Trudel M. Coliphages and enteric viruses in the particulate phase of river water. Can J Microbiol. 1988 Jul;34(7):907-10.

Peng YH, Nishizawa T, Takahashi M, Ishikawa T, Yoshikawa A, Okamoto H 2002. Analysis of the complete genomes of thirteen TT virus variants classifiable into the fourth and fifth genetic groups, isolated from viremic infants. Arch Virol 147:21-41.

Prescott LE, Simmonds P. Global distribution of transfusion-transmitted virus. New Engl J Med. 1998 Sep:339(11):776-7.

Prescott, L. E., D. M. MacDonald, F. Davidson, J. Mokili, D. I. Pritchard, D. E. Arnot, E. M. Riley, B. M. Greenwood, S. Hamid, A. A. Saeed, M. O. McClure, D. B. Smith, P. Simmonds. 1999. Sequence diversity of TT virus in geographically dispersed human populations. J Gen Virol. 80: 1751–8.

Projeto Geocidades - relatório ambiental urbano integrado. Informe GEO: Manaus (2002). Rio de Janeiro: Consórcio parceria 21. 188p.

Pusch D, Oh DY, Wolf S, Dumke R, Schröter-Bobsin U, Höhne M, Röske I, Schreier E. Detection of enteric viruses and bacterial indicators in German environmental waters. Arch Virol. 2005 May;150(5):929-47.

Qiu J, Kakkola L, Cheng F, Ye C, Söderlund-Venermo M, Hedman K, Pintel DJ. Human circovirus TT virus genotype 6 expresses six proteins following transfection of a full-length clone. J Virol. 2005 May;79(10):6505-10.

Queiroz AP, Santos FM, Sassaroli A, Hársi CM, Monezi TA, Mehnert DU. Electropositive filter membrane as an alternative for the elimination of PCR inhibitors from sewage and water samples. Appl Environ Microbiol. 2001 Oct;67(10):4614-8.

Romeo, R, P. Hegerich, S. U. Emerson, M. Colombo, R. H. Purcell, J. Bukh. 2000. High prevalence of TT virus (TTV) in naive chimpanzees and in hepatitis C virus-infected humans: frequent mixed infections and identification of new TTV genotypes in chimpanzees. J Gen Virol. 81:1001–7.

Rose JB, Gerba CP. Use of risk assessment for development of microbial standards. Water Sci Technol. 1991;24(2):29-34.

Ross RS, Viazov S, Runde V, Schaefer UW, Roggendorf M. Detection of TT virus DNA in specimens other than blood. J Clin Virol. 1999 Aug;13(3):181-184.

Saback FL, Gomes SA, de Paula VS, da Silva RR, Lewis-Ximenez LL, Niel C. Age-specific prevalence and transmission of TT virus. J Med Virol. 1999 Nov;59(3):318-322.

Schvoerer E, Bonnet F, Dubois V, Cazaux G, Serceau R, Fleury HJ, Lafon ME. PCR detection of human enteric viruses in bathing areas, waste waters and human stools in Southwestern France. Res Microbiol. 2000 Oct;151(8):693-701.

Schvoerer E, Ventura M, Dubos O, Cazaux G, Serceau R, Gournier N, Dubois V, Caminade P, Fleury HJ, Lafon ME. Qualitative and quantitative molecular detection of enteroviruses in water from bathing areas and from a sewage treatment plant. Res Microbiol. 2001 Mar;152(2):179-86.

Shuval HI. Detection and control of enteroviruses in the water environment. In: Shuval HI. Developments in Water Quality Research. Ann Arbor: Ann Arbor-Humphrey 1971:47.

Simmonds P, Davidson F, Lycett C, Prescott LE, MacDonald DM, Ellender J, Yap PL, Ludlam CA, Haydon GH, Gillon J, Jarvis LM. Detection of a novel DNA virus (TTV) in blood donors and blood products. Lancet. 1998 Jul 18;352(9123):191-5.

Straub TM, Chandler DP. Towards a unified system for detecting waterborne pathogens. J Microbiol Methods. 2003 May;53(2):185-97.

Takahashi K, Iwasa Y, Hijikata M, Mishiro S. Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. Arch Virol. 2000;145(5):979-93.

Takahashi K, Ohta Y, Mishiro S. Partial 2.4-kb sequences of TT virus (TTV) genome from eight Japanese isolates: diagnostic and phylogenetic implications. Hepatol Res. 1998;12(2):111-20.

Tawara A, Akahane Y, Takahashi M, Nishizawa T, Ishikawa T, Okamoto H. Transmission of human TT virus of genotype 1a to chimpanzees with fecal supernatant or serum from patients with acute TTV infection. Biochem Biophys Res Commun. 2000 Nov 19;278(2):470-6.

Tree JA, Adams MR, Lees DN. Chlorination of indicator bacteria and viruses in primary sewage effluent. Appl Environ Microbiol. 2003 Apr;69(4):2038-43.

Ukita M, Okamoto H, Kato N, Miyakawa Y, Mayumi M. Excretion into bile of a novel unenveloped DNA virus (TT virus) associated with acute and chronic non-A-G hepatitis. J Infect Dis. 1999 May;179(5):1245-8.

Umemura T, Alter HJ, Tanaka E, Yeo AE, Shih JW, Orii K, Matsumoto A, Yoshizawa K, Kiyosawa K. Association between SEN virus infection and hepatitis C in Japan. J Infect Dis. 2001 Nov 15;184(10):1246-51.

Vaidya SR, Chitambar SD, Arankalle VA. Polymerase chain reaction-based prevalence of hepatitis A, hepatitis E and TT viruses in sewage from an endemic area. J Hepatol. 2002 Jul;37(1):131-6.

Van Heerden J, Ehlers MM, Van Zyl WB, Grabow WO. Incidence of adenoviruses in raw and treated water. Water Res. 2003 Sep;37(15):3704-8.

Verani M, Casini B, Battistini R, Pizzi F, Rovini E, Carducci A. One-year monthly monitoring of Torque teno virus (TTV) in river water in Italy. Water Sci Technol. 2006;54(3):191-5.

Verschoor EJ, Langenhuijzen S, Heeney JL. TT viruses (TTV) of non-human primates and their relationship to the human TTV genotypes. J Gen Virol. 1999 Sep;80:2491-9.

Viazov S, Ross RS, Niel C, de Oliveira JM, Varenholz C, Da Villa G, Roggendorf M. Sequence variability in the putative coding region of TT virus: evidence for two rather than several major types. J Gen Virol. 1998 Dec;79(Pt 12):3085-9.

Wallis C, Melnick JL. Concentration of viruses on aluminum and calcium salts. Am J Epidemiol. 1967 May;85(3):459-68.

Wyn-Jones AP, Sellwood J. Enteric viruses in the aquatic environment. J Appl Microbiol. 2001 Dec;91(6):945-62.

Yazici M, Comert MR, Mas R, Guney C, Cinar E, Kocar IH. Transfusion-transmitted virus prevalence in subjects at high risk of sexually transmitted infection in Turkey. Clin Microbiol Infect. 2002 Jun;8(6):363-7.

Zheng H, Ye L, Fang X, Li B, Wang Y, Xiang X, Kong L, Wang W, Zeng Y, Ye L, Wu Z, She Y, Zhou X. Torque teno virus (SANBAN isolate) ORF2 protein suppresses NF-kappaB pathways via interaction with IkappaB kinases. J Virol. 2007 Nov;81(21):11917-24.

Zhong S, Yeo W, Lin CK, Lin XR, Tang MW, Johnson PJ. Quantitative and genotypic analysis of TT virus infection in Chinese blood donors. Transfusion 2001 Aug;41(8):1001-7.

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