

## LONGITUDINAL STUDY ON OCCURRENCE OF ADENOVIRUSES AND HEPATITIS A VIRUS IN RAW DOMESTIC SEWAGE IN THE CITY OF LIMEIRA, SÃO PAULO

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### ABSTRACT

The aim of this study was to verify the presence and annual distribution of adenoviruses and hepatitis A virus in domestic sewage in the city of Limeira, São Paulo. Fifty samples with a volume of 8 liters each were collected weekly from December 2004 to December 2005. The viruses were concentrated by filtration through positively charged ZP60S filter membranes, followed by ultracentrifugation. Human adenoviruses (HAdV) were detected by PCR followed by nested-PCR and screening for species F was done by restriction of the PCR product with *TaqI* endonuclease. Virus infectivity assays were performed by inoculation of concentrates onto HEp-2 cell monolayers. RT-PCR was used for the detection of hepatitis A virus. HAdV were detected in all samples, and 64% of samples were positive for infectious virus. Species F was present in 82% of the samples. Hepatitis A virus was detected in 48% of the samples. These results demonstrate that HAdV and HAV were present in the domestic sewage of Limeira throughout the period of study, demonstrating the importance of an adequate treatment before the disposal in the environment.

**Key-words:** adenoviruses, human adenovirus F, hepatitis A virus, sewage treatment.

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Domestic sewage contains pathogenic organisms released in feces from infected people and may contaminate the environment if not properly treated. In Brazil, only 51.6% of urban populations and 3.7% of rural populations have sewage collection systems, while cesspools are used in 23.3% of urban populations and 12.3% of rural populations (12). Almost 33% of rural residents and 3% of urban residents do not have any type of sewage collection system. From the amount of sewage collected, only 33% is submitted to any type of treatment, with the remaining sewage discarded untreated on soils or rivers (12). These sanitary conditions are directly related to the population's lack of health (11,12). A wide variety of pathogenic microorganisms are present in sewage and its release in waters contaminates the environment and infects the people who make use of them. Among the pathogens, there are 150 types of human enteric viruses transmitted by the fecal-oral route and some are excreted in great numbers,

around  $10^8$  to  $10^{11}$  particles/gram of feces (28,35). These viruses cause diseases like gastroenteritis, hepatitis, conjunctivitis, meningitis, among others. Their epidemiology is influenced by the level of hygiene and sanitation conditions of the population (28,31). Despite all this data, only few studies have been conducted in Brazil to evaluate the presence and distribution of human enteric viruses in domestic sewage (5,7,19-22,26,29,30,32,36).

Among the enteric viruses, the human adenoviruses (HAdV) are responsible for persistent infections and outbreaks in drinking and recreational waters (14,16). At the present, the enteric adenoviruses of specie F, HAdV-40 and HAdV-41, are the third cause of non-bacterial diarrhea among young children (3,9). HAdV-41 is fatal in 50% of immunosuppressed individuals infected (11). The resistance of HAdV-40 and HAdV-41 to inactivation by UV light has already been demonstrated (15,34). The recent addition of HAdV in EPA's contaminant candidate

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list indicates a high priority for a possible recommendation of their use as an indicator of water quality (18).

Hepatitis A viruses (HAV) are responsible for outbreaks worldwide, with 1.4 million cases per year, affecting mainly children and adults older than 50 years (38). The release of viruses in feces occurs from 3 to 10 days before the establishment of clinical symptoms and up to 8 days after the manifestation of jaundice (10). HAV epidemiology is greatly influenced by the level of sanitation and hygiene. Infections occur during childhood and almost 100% of children acquire immunity until the age of ten (10,38). The improvement of sanitation and hygiene in some countries has changed this incidence, showing now a greater susceptibility of young adults (33). Like HAdV, studies have also demonstrated the occurrence of HAV in domestic sewage (24,30). Although the HAV is a simple positive strand RNA virus, it is highly resistant to thermic denaturation, treatment with acids, chloroform, perchloroacetic acid and detergents (10,38).

Considering the possibility of HAdV and HAV being included in international and Brazilian regulations as better parameters than bacteria to evaluate the efficiency of sewage treatment due to their greater resistance, the aim of the present study was to evaluate the occurrence and distribution of HAdV, including the species F, and HAV in the domestic sewage of the city of Limeira, SP, throughout a year.

## MATERIAL AND METHODS

### Samples collection

A total of fifty samples of raw domestic sewage were collected weekly at the sewage treatment plant in the city of Limeira, São Paulo, from December 2004 to December 2005. Eight-liter samples were collected and immediately processed.

### Sample processing

Viruses were concentrated by filtration through positively charged ZP60S (AMF, Cuno Div., Meridenn, Conn.) filter membranes and subsequent ultracentrifugation as described by Mehnert *et al.* (21). Detoxification of concentrates was done by organic extraction using Vertrel-XF (1, 1, 1, 2, 3, 4, 4, 5, 5, 5, 5, 5 - decafluoropentane, Clarus®, Hortolândia, SP) as previously described (27).

For the molecular assays, the viral nucleic acid (DNA for HAdV and RNA for HAV) extraction was performed using Trizol™ (Invitrogen, Carlsbad, California), as recommended by the manufacturer. For viral infectivity assays, 500 µL aliquots of samples were treated with antibiotics (1000 IU of penicillin G/mL and 1,000 µg/mL of streptomycin) (19). All samples were stored in -20°C or -70°C (RNA) until their use.

### Cell cultures and viruses

The viruses were cultivated in specific cell culture lines: HEP-2 (human epidermoid carcinoma of larynx) for HAdV-5;

HEK-293 (human kidney embryonic cell line, transformed with HAdV-5) for adenovirus F and FRhK-4 (fetal rhesus kidney cell) for HAV (HM-175 strain). All cell lines were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Emcare, Campinas, Brazil) and antibiotics (100U of penicillin G/mL and 100 µg/mL of streptomycin).

Control strains of human adenoviruses C (HAdV-5) and F were kindly provided by Prof. Charlotte M. Hársi. For the detection of HAV, the control strain HM-175, was kindly provided by Prof. Ana Maria Gaspar, Department of Virology, FIOCRUZ. Control strains were inoculated onto 75% confluent monolayers of either cell line and incubated for 48 to 72 hours at 37°C in a 5% CO<sub>2</sub> atmosphere.

PCR and RT-PCR. For the detection of HAdV by PCR the primers hexAA1885 and hexAA1913, for the amplification of a 301 bp fragment of the *hexon* protein gene were used (2). The HAdV PCR was performed as described by Allard *et al.* (2), with modifications by Santos *et al.* (29).

The samples that were negative at the first amplification had their product subjected to a second round of amplification (nested-PCR) using primers nehexAA1893 and nehexAA1905 which amplify a 143 bp fragment (3).

The detection of HAV was performed using the primers HAV-CL and HAV-CR of De Leon *et al.* (6), which correspond to the region between the coat protein VP1 and VP3 genes and amplify a 207 bp fragment. The RNA genome of the viruses was reverse transcribed into cDNA using the Moloney murine leukemia virus reverse transcriptase (M.M.L.V.; Invitrogen) and the respective pair of primers (6). The RT-PCR was done following the procedure described by De Leon *et al.* (6) and modified by Sassaroli (30).

All the PCR runs included ultrapure water (Milli-Q®) treated with 0.01% diethylpyrocarbonate (DEPC) as negative controls. Samples that did not show amplification of naturally occurring adenoviruses were seeded with 5 µL of viral DNA and reamplified by PCR to evaluate the interference by inhibitors.

Amplification products were analyzed by 1.5% agarose gel electrophoresis in TAE buffer and ethidium bromide (0.5 µg/mL) staining.

HAdV species F characterization. The distinction of enteric adenoviruses (species F) from the other species was performed by restriction fragment length polymorphism (PCR-RFLP). The digestion of the 301 bp amplicons was performed by using restriction endonuclease *TaqI* (Fermentas®), following procedures described by Santos *et al.* (29). Restriction profiles were analyzed by 1.5% agarose gel electrophoresis in TAE buffer and ethidium bromide (0.5 µg/mL) staining.

Infectivity assays. Samples were subcultured three times in HEP-2 cells for virus isolation. Duplicate cell monolayers were inoculated with 100 µL of each sample. Cultures were incubated at 37°C in 5% CO<sub>2</sub> for 10 days and observed daily for cytopathic effect (CPE).

## RESULTS AND DISCUSSION

Among the enteric viruses, adenoviruses and hepatitis A viruses have their importance for Public Health due their resistance and high rate of outbreaks worldwide (8,10,15,18, 25,34). In Brazil there is a lack of information on the occurrence of human enteric viruses in domestic sewage and its correlation with possible outbreaks. Most of data were obtained from large cities like São Paulo (5,7,19-22,26,29, 30,32,36) and there is no data available from rural areas of São Paulo state.

The city of Limeira was chosen for this study due to its geographical and demographical aspects. Located in the countryside of São Paulo state, it has 272.734 inhabitants (13) and the collection and treatment of both water and sewage are done by a private company, Águas de Limeira S/A. At present, the company collects 100% of domestic sewage and treats 77% of it (4). This is a high percentage when compared to the country's average, where 53% of sewage is collected and 34% treated (12). It is not uncommon to find illegal discharges of industrial effluents that may adversely affect the treatment. There are three sewage treatment plants and Tatu is the largest one, with a primary treatment that reduces 40% of biological oxygen demand before discharging in the Tatu river (1).

Adenoviruses. Of the 50 samples of domestic sewage, 42 (84%) samples were positive after the first round of PCR. Eight (16%) additional samples were positive for adenoviruses after nested-PCR, showing that the viruses were present in low concentrations in these samples.

The characterization of species F by RFLP was based on the presence of a restriction site for the endonuclease *TaqI* located in the *hexon* gene sequence. The amplicons from HAdV-40 and HAdV-41 present two restriction fragments of 191bp and 110bp after the digestion, while the adenoviruses from other species (HAdV-A to E) do not (3,29). Based on this assay, the presence of human adenovirus from species F was detected in 41 (82%) samples and only 1 (2%) sample did not show human adenovirus F. Eight (16%) samples were not characterized due to low DNA concentration for the restriction assay. Of the 42 samples submitted to characterization, 40 (92,9%) samples presented non-restricted fragments of 301 bp, revealing the presence of the other serotypes of adenoviruses. This result is not a surprise, because all the other serotypes are also excreted in feces.

Viral infectivity assays revealed that 32 (64%) of the samples contained infectious adenoviruses. These results may be underestimated due to the great diversity of other enteric viruses in those samples, like enterovirus, which have a faster replicative cycle than adenoviruses, damaging the cells just a few hours after infection (data not shown).

The high occurrence of adenoviruses and the diversity of species in raw domestic sewage agree with the results from

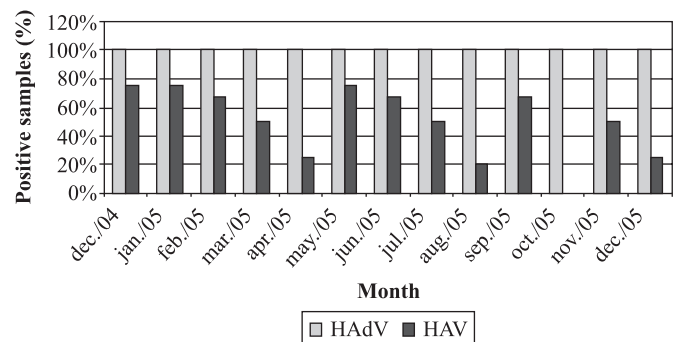
previous studies in the cities of Barcelona (23) and São Paulo (29). Although the molecular techniques can not distinguish infectious from non infectious viruses, the infectivity assays showed that most of the samples contained infectious adenoviruses. As most samples had adenoviruses detected at the first PCR, which detection limit is  $10^{-4}$  DICT<sub>50</sub> as previously established by Santos *et al.* (29), the results suggest a high concentration of adenoviruses in sewage.

Hepatitis A. Of the 50 samples subjected to the RT-PCR reaction for HAV detection, 24 (48%) samples were positive. These results are similar to those previously observed for raw domestic sewage of the city of São Paulo (30). Although the RT-PCR can not distinguish between infectious and non-infectious particles, in the case of single strand RNA viruses like HAV, a positive result in RT-PCR suggests the recent presence of potentially infectious viruses in the sewage. The viruses detected by RT-PCR were considered infectious, because the viral genome is infectious *per se*, and rapidly degraded after the release of the capsid (17). The detection of HAV in 48% of samples also demonstrates the stability of the virus in the environment.

The occurrence of both types of enteric viruses in the domestic sewage of Limeira throughout this study is shown in Table 1 and their annual distribution in Fig. 1.

Both viruses did not show a seasonal distribution in the domestic sewage of Limeira during the period of study and all samples showed at least one type of virus. The absence of HAV in October and a low positivity in August, November and December may be due to the destruction of the viral particles due to illegal discharge of industrial effluent in the domestic sewage (Águas de Limeira, data not shown).

The data demonstrate a continuous release of HAdV and HAV by the population of Limeira and are similar to other studies on detection of enteric viruses in sewage, not only in Brazil, but also in Spain (7,17,19,20,22-26,29,30,32,36). The persistence of infectious viruses and the absence of seasonality emphasize



**Figure 1.** of HAdV and HAV positive domestic sewage samples from December 2004 to December 2005.

**Table 1.** Detection of HAdV and HAV in the domestic sewage of Limeira from December 2004 to December 2005 using molecular and cell culture methods.

Month	No. of samples examined	Detection methods						
		Adenoviruses					HAV	
		PCR	Nested-PCR	CC	RFLP			RT-PCR
F+ others	Only Species F				Only other species			
Dec./04	4	2	2	1	1	ND	1	3
Jan./05	4	4	NT	2	4	ND	ND	3
Feb./05	3	3	NT	2	3	ND	ND	2
Mar./05	4	4	NT	3	4	ND	ND	2
Apr./05	4	2	2	4	2	ND	ND	1
May./05	4	1	3	3	1	ND	ND	3
Jun./05	3	3	NT	3	3	ND	ND	2
Jul./05	4	3	1	2	3	ND	ND	2
Aug./05	5	5	NT	2	5	ND	ND	1
Sep./05	3	3	NT	2	3	ND	ND	2
Oct./05	4	4	NT	3	3	1	ND	ND
Nov./05	4	4	NT	2	3	1	ND	2
Dec./05	4	4	NT	3	4	ND	ND	1
Total	50	42	8	32	39	2	1	24
%		84%	16%	64%	78%	4%	2%	48%

CC = cell culture; NT = Not tested due to detection on first PCR; ND = not detected.

their importance to public health. Considering the low infective dose of these viruses that may range from 10 to 100 particles (37), the release of domestic sewage in rivers without adequate treatment is not recommended, as it may be a source of dissemination of hepatitis A, diarrhea, gastroenteritis, respiratory infectious diseases, among others (11,16).

The viral infections may harm not only the health of people who live in places where there is a lack or absence of sanitation, but it also harms the local economy, when employees are ill and unable to work (38). An increase in the number of sewage treatment plants during the last few years (4) and the possibility to use sewage by-products (biosolids) in agriculture reinforces the importance of systematic studies of the viruses circulating in sewage in different regions of the country (28,31).

The results of the study may be used as a source of information to justify the importance of implementing effective sewage treatment plants to inactivate viral particles before final disposal in the environment.

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#### RESUMO

##### Estudo longitudinal da ocorrência de adenovírus e vírus da hepatite A em esgoto doméstico na cidade de Limeira, SP

O objetivo do estudo foi verificar a ocorrência e a distribuição anual de adenovírus humanos e vírus da Hepatite A (VHA) no efluente doméstico da cidade de Limeira, São Paulo, ao longo do período de Dezembro de 2004 e Dezembro de 2005, com vistas à futura implementação de sistemas de tratamento de água de esgoto. Cinquenta amostras de efluente bruto com volume de 8L cada foram colhidas semanalmente e os vírus concentrados por filtração em membrana eletropositiva ZP60S, seguida de ultracentrifugação. Adenovírus foram detectados por PCR e *nested*-PCR. Adenovírus da espécie F foram distinguidos das demais por restrição do produto da PCR com endonuclease *TaqI*. Ensaio de infectividade viral foram realizados em culturas de

células HEp-2. A presença do vírus da hepatite A também foi pesquisada nas mesmas amostras, fazendo-se uso de método de RT-PCR. Adenovírus foram detectados em todas as amostras, sendo a espécie F identificada em 82% destas. Sessenta e quatro por cento dos adenovírus detectados ainda estavam infecciosos. O vírus da Hepatite A foi detectado em 48% das amostras examinadas. Estes resultados evidenciam a presença e a circulação de Adenovírus humano e VHA nas águas de esgoto doméstico de Limeira ao longo do período de estudo, demonstrando a importância de um tratamento adequado desse material antes da disposição no meio ambiente.

**Palavras-chave:** adenovírus, adenovírus espécie F, vírus da hepatite A, efluente doméstico.

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