Analysis of the evolution in the circulation of HAV and HEV in Eastern Spain by testing urban sewage samples

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ABSTRACT

The aim of the study was to analyse the evolution of the prevalence of HAV and HEV in the population of eastern Spain by analysing the viruses excreted in urban sewage. Raw urban sewage samples were collected and analysed during several years using RT-PCR techniques and sequencing analysis. Two limiting regions were analysed, one of them having implemented HAV vaccination programs. Acute symptomatic HEV cases were also examined. Results were compared with those from previous studies in the area using identical methodology. The percentage of positive HAV samples in urban sewage fell from 57.4% to 3.1% in 5–10 years in the two studied areas in Spain. Around 30% of the urban sewage samples were positive for HEV in the absence of agricultural sources of contamination. HEV RNA was also detected in four clinical cases of acute hepatitis. The dramatic reduction in the presence of HAV in raw urban sewage observed in eastern Spain could be most likely related to the general improvement in sanitation. However, these improvements would not have an equivalent effect on the circulation of HEV and this observation could be explained by the presence of animal reservoirs for HEV, which act as external sources of infections.

Key words | acute hepatitis, HAV, HEV, sanitation, vaccination, wastewater

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ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic acid
EIA	Enzyme immunoassay
EMEM	Eagle's minimum essential medium
FDA	Food and Drug Administration
GC	Genomic copies
HAdV	Human adenovirus
HAV	Hepatitis A virus
HBeAg	Hepatitis B e-antigen
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C virus
HEV	Hepatitis E virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
NAs	Nucleic acids
NCBI	National Center for Biotechnology
NE	Northeast
NJ	Neighbour joining

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ORF2	Open Reading Frame 2
PCR	Polymerase Chain Reaction
QPCR	Quantitative PCR
RNA	Ribonucleic acid
RT–PCR	Reverse Transcriptase PCR
VP1/2A	Viral Protein 1/2A

INTRODUCTION

Hepatitis A virus (HAV) and hepatitis E virus (HEV) are small, non-enveloped viruses that contain positive sense, single-stranded RNA genomes of 7.5 and 7.2 kb, respectively (Purcell & Emerson 2008). HAV has its own genus (*Hepatovirus*) within the family *Picornaviridae* whereas HEV is the sole member of the genus *Hepevirus* and family *Hepeviridae* (Emerson *et al.* 2005; Nainan *et al.* 2006). Both viruses cause acute, self-limited infections that may vary in severity from asymptomatic to fulminant (Purcell & Emerson 2008). The most common route of transmission is generally faecal-oral (Orrù *et al.* 2004) and it causes large-scale epidemics.

Hepatitis A affects humans worldwide. HAV infection is endemic to developing countries where most individuals are exposed to this virus during childhood (Jacobsen & Koopman 2004). Hepatitis E is the major cause of waterborne outbreaks and sporadic cases of acute hepatitis in many developing countries where sanitation is suboptimal and where HEV is considered endemic (Clemente-Casares *et al.* 2003; Emerson & Purcell 2004). Typically, the disease caused is self-limited but it sometimes has severe complications, particularly in pregnant women, who register a high mortality rate (20%) (Balayan 1997).

Traditionally, North America and Europe have been considered non-endemic regions for HEV as they have seroprevalence rates of 1 to 5% and most HEV infections are thought to be imported (Paul *et al.* 1994). However, in the last few years several HEV strains associated with sporadic acute hepatitis and genetically distinct from the endemic ones have been isolated from human serum samples in North America and in some European countries (Clemente-Casares *et al.* 2009; Legrand-Abravanel *et al.* 2009). A recent study conducted in the same area, Catalonia (NE Spain), showed a seroprevalence of about 7.3% (Buti *et al.* 2006). There is also evidence that some animals, particularly swine, are infected by HEV genotypes 3 and 4. Both genotypes have been recovered from swine principally in the same regions that these genotypes were recovered from humans (Meng *et al.* 1997; Purcell & Emerson 2008). With few exceptions, genotypes 3 and 4 appear not to produce disease in swine (Martín *et al.* 2007). In certain regions, including Spain, swine and human HEV strains are not genetically distinguishable (Clemente-Casares *et al.* 2009), thereby supporting the notion that swine are a reservoir and that HEV infection is acquired by handling these animals or eating raw pork products.

Spain is considered an area of low endemicity for HAV infection (Bell 2002) and is characterized by diminishing HAV seroprevalence in the population (Jacobsen & Koopman 2004), while it is a non-endemic area for HEV (Yarbough 1999). Previous studies in Spain show that even in the absence of significant numbers of clinical cases of acute hepatitis A and E, HAV and HEV are present in more than 50 and 40%, respectively, of urban sewage samples analysed, thereby indicating a high level of subclinical infection in the population (Pina *et al.* 2001; Clemente-Casares *et al.* 2003).

In 2001, the FDA approved a new combination vaccine against HAV and the hepatitis B virus. The vaccine, called Twinrix, contains the approved vaccine Havrix® (Hepatitis A Vaccine, Inactivated) (US Food and Drug Administration 2001). Twinrix has been distributed in a pilot program in Catalonia for pre-adolescents since 1999. In Valencia, a region immediately south of Catalonia, HAV vaccines are distributed only among groups at risk as a result of handling food. No HEV vaccine is currently available for humans.

By 2006, 235,170 and 11,826 doses of vaccines against HAV had been distributed in Catalonia (covering around 90% of pre-adolescents) and Valencia (in the professional food handler group), respectively (Departament de Salut, Generalitat de Catalunya 2008; Direcció general de salut pública, conselleria de sanitat, Generalitat Valenciana 2008).

This study analyses changes in the excretion patterns of HAV and HEV in eastern Spain by testing the presence of these viruses in urban sewage over several years in order to evaluate the potential impact of improvements in sanitation

and vaccination programs for HAV. Serum samples from acute hepatitis patients were also analysed to confirm the presence of HEV strains in the population.

METHODS

Sewage samples

Sampling areas were selected on the basis of the vaccination programs applied for HAV. Samples were collected in Barcelona and Valencia, the largest cities in Catalonia and Valencia respectively, both in Spain. A total of 91 urban sewage samples were collected in Barcelona (50 samples) and Valencia (41 samples) from 2000 to 2008. Samples were collected at the entry of two treatment plants in Catalonia receiving sewage from a population of about 1.8 million (Barcelona) and three plants from the area of Valencia, with a total population of about 800,000 inhabitants. Each sample was harvested in a sterile 500-ml polyethylene container and kept at 4°C for less than 8 h until the virus particles were concentrated.

Clinical samples

From 2004 to 2007, 19 serum samples were collected from patients with symptomatic acute hepatitis attending the Hospital General Valle Hebron (Barcelona, Spain). All patients had elevated aminotransferase levels (>10 times the upper normal limit) and IgG anti-HEV antibodies. Clinical serum samples were also analysed for other viral infections. Hepatitis B surface antigen and antibody to HCV (anti-HCV) were detected by commercial enzyme immunoassays (Vitros HBsAg and Vitros anti-HCV; Johnson & Johnson, Rochester, NY, USA). IgM anti-HBc and IgM anti-HAV were detected by enzyme immunoassays (Liaison HBeAg/anti-HBe, Liaison HBc IgM and Liaison HAV IgM, respectively; Diasorin, Vercelly, Italy). IgG anti-HEV was the routine assay in use for HEV infections and was determined by EIA (Bioelisa HEV IgG; Biokit, Barcelona, Spain). Serum samples were stored at −80°C until use for detecting genomic sequences. Other causes of acute hepatitis such as autoimmune hepatitis or biliary stones were excluded.

Viruses

The positive controls used for the analysis of HAV consisted on dilutions of the supernatant of FRhK-4 cell cultures infected with pHM-175 strain. As positive controls for HEV analysis, 10% faecal suspensions obtained from rhesus monkeys experimentally infected with a strain isolated from a sewage sample collected in Barcelona (Pina *et al.* 1998) were used. HAdV2, isolated from a clinical sample and used as positive control for the determinations of human adenoviruses, was grown on A549 cells propagated in Eagle's minimum essential medium (EMEM) supplemented with 1% glutamine, 50 μg of gentamicin per ml, and 5% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS. Viral suspensions were stored at −80 °C until use.

Concentration of viruses from urban sewage

The method used to recover virus particles from sewage samples was chosen on the basis of previous studies (Puig *et al.* 1994; Pina *et al.* 1998). Briefly, 42 ml of sewage was ultracentrifuged at 110,000 × *g* for 1 h at 4°C to pellet all the viral particles together with any suspended material. The pellet was eluted by mixing it with 3.5 ml of 0.25 N glycine buffer (pH 9.5) on ice for 30 min and, after addition of 3.5 ml of 2X phosphate-buffered saline, the suspended solids were separated by centrifugation at 12,000 × *g* for 20 min. Finally, viruses were concentrated by ultracentrifugation at 110,000 × *g* for 1 h at 4°C, resuspended in 100 μl of phosphate-buffered saline and stored at −80°C.

Nucleic acid extraction

The nucleic acids (NAs) from viral concentrates obtained from sewage samples were extracted following a highly sensitive and efficient procedure (Puig *et al.* 1994) described by Boom *et al.* (Boom *et al.* 1990). This procedure is based on the use of guanidinium isothiocyanate and silica particles.

For clinical serum samples, QIAamp RNA Blood Mini Kit® (Qiagen, Valencia, Spain) was used for the viral RNA extraction, following the manufacturer's instructions.

Reverse transcription-polymerase chain reaction

All samples were analysed with primers designed to amplify a region within the ORF2 of HEV (Erker *et al.* 1999) (semi-nested RT-PCR) and a non-codifying region of 5′ end of HAV (Pina *et al.* 2001) (nested RT-PCR). The VP1/2A region was used to characterize HAV-positive samples by sequencing (Pina *et al.* 2001). Positive and negative controls extracted in parallel and inhibition controls were added to the PCR assays. The results of the PCRs using OneStep® RT-PCR kit from Qiagen, for a typical one-step reaction, and the 50-μl reaction mixture containing PCR Gold Buffer, 1.2 mM $MgCl_2$ and 2 U of AmpliTaq® Gold (Applied Biosystems, New Jersey, USA), for a second PCR amplification cycle, were analysed by electrophoresis on agarose gels to 3% (w/v), followed by staining with ethidium bromide at 0.5 μg/ml. The amplicons were viewed using an Image Master® VDS (Pharmacia Biotech, Uppsala, Sweden).

Analysis of sewage samples by QPCR

HAV- and HEV-positive samples by nested PCR were quantified by real-time PCR (Jothikumar *et al.* 2005*b*, 2006). Five microlitres of the extracted RNA, corresponding to 2.1 ml sewage, was analysed. Five microlitres of ten-fold dilutions (from 5×10^0 to 5×10^7 GC/5 μl) of a plasmid suspension were used as standard. Amplification reactions were performed with the QuantiTect™ Probe RT-PCR kit reagents (Qiagen).

Human adenoviruses (HAdVs) were determined in each location. The viral load of HAdV was used to discard negative HAV and HEV results caused by the absence of human faecal contamination, which could influence the results of the study (Bofill-Mas *et al.* 2006).

QPCR amplification reactions for HAdV were carried out using TaqMan® Universal PCR Master Mix reagents (Applied Biosystems), as previously described (Jothikumar *et al.* 2005*a*; Bofill-Mas *et al.* 2006). The HAdV 41 used in the construction of the standards was kindly donated by Dr Annika Allard at the University of Umeå, Sweden. Ten microlitres of the NA extractions were analysed, corresponding to 4.2 ml of sewage sample. Dilutions from 10^{-1} to 10^7 CG/10 μl of the standard were analysed in triplicate.

In all PCR assays, standard precautions were taken by using separate areas for the diverse steps of the protocols. Negative controls, inhibition controls and positive controls were added in each assay. Positive results were confirmed by sequencing analysis of the amplified DNA. The QPCRs were conducted in a thermocycler Stratagene® MX3000P (Stratagene, La Jolla, USA). All the samples and their respective dilutions were analysed in duplicate.

Sequencing and analysis of viral genomes

Amplicons obtained with the nested PCR were purified using a QIAquick® purification kit (Qiagen), following the manufacturer's instructions. Both strands of the purified DNA amplicons were sequenced using an ABI Prism 3100 Genetic Analyzer and Big Dye Terminator Cycle Sequencing Kit v. 3.1® (Applied Biosystems), following the manufacturer's instructions. Products were checked using an ABI PRISM 377 analyser (Applied Biosystems) by the Scientific and Technical Services of the University of Barcelona. The sequences were compared with the nucleotide sequences present in the GenBank using the BLAST program of the NCBI (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov/BLAST) and aligned using the Clustal X 1.8 program. Phylogenetic tree reconstructions were performed with Mega 3.1 (www.megasoftware.net). Evolutionary distances were determined by the Kimura two-parameter equation, and the tree was constructed by using the neighbour-joining (NJ) algorithm. The GenBank accession numbers for the sequences reported in this study are FJ010829-FJ010840 (HAV in sewage), FJ010841-FJ010844 (HEV in serum samples) and FJ010845-FJ010858 and EU729700 (HEV in sewage).

RESULTS AND DISCUSSION

This study was designed as a comparative analysis for the evaluation of changes in excretion patterns of acute hepatitis viruses in eastern Spain. Previous studies conducted in our laboratory between 1994 and 2002 analysed environmental and clinical samples using the same protocols as in this study and reported a frequent presence of HAV and HEV in urban sewage (Pina *et al.* 2001;

Clemente-Casares *et al.* 2003). The recent availability of an efficient vaccine against HAV infections, the implementation of vaccination programs for a high proportion of the susceptible population and a general improvement in sanitary conditions are expected to decrease the circulation of the virus in the population and the levels of excretion of HAV in the urban sewage generated.

Presence and concentration of HAV and HEV

Average viral concentrations in wastewater, as determined by the QPCR assays, were 1.6×10^2 GC/ml for HAV and 3.2×10^1 GC/ml for HEV. The results on the prevalence of positive HAV and HEV samples using RT-PCR assays were equivalent in the two regions in NE Spain (Table 1).

HEV was detected in clinical serum samples using nested RT-PCR. Four of the 19 analysed serum samples analysed were positive for HEV RNA, with one sample also being positive for IgM anti-HAV and one positive for anti-HCV antibodies without HCV RNA. These results confirm that HEV circulates in the population and that it causes sporadic clinical cases of acute hepatitis.

In 2001, Pina *et al.* (Pina *et al.* 2001), using the same methodology as in the present study, analysed HAV strains excreted in sewage in the area of Barcelona and also the HAV strains responsible for acute hepatitis A clinical infections in Barcelona. These authors found that 57.4% (31/54) of the sewage samples collected from 1994 to 2000 were positive for HAV and identified clinical HAV infections. In addition, the distribution of HAV was found to be equivalent to the strains detected in urban sewage. That study is considered a reference for the evaluation of changes in the epidemiological pattern of HAV in the study area.

The results obtained in this study show a marked reduction in the prevalence of HAV in the study area, thereby suggesting that the higher risk of this population to acquire HAV infection is at present through personal or food-borne transmission from other areas with a higher level of endemicity.

General immunization in adolescents has a protective effect on the population; however, the pattern of HAV excretion in urban sewage did not differ significantly between the two regions in Spain, one of which has a general vaccination program while the other does not. However, what is unquestionable is that vaccination is the only measure that guarantees individual protection against HAV infections in susceptible populations.

Improvements in sanitation would include not only improvements in water treatment but also improvements in hygienic and preventive practices that will better protect the environment, water and food from contamination, reducing the overall exposure and the number of infections and excreted viruses in the population. HAV is known to be a very stable virus (Siegl *et al.* 1984). The samples analysed in this study were raw urban sewage samples presenting standard concentrations of human adenoviruses and without any treatment (Bofill-Mas *et al.* 2006). The observed differences in the presence of HAV in the samples would be then related with higher probability to the reduction in the prevalence and excretion of hepatitis A viruses in the population than to significant changes in the stability of the viruses or in the level of faecal contamination in the sewage.

Table 1 | Presence of hepatitis A virus (HAV) and hepatitis E virus (HEV) in urban wastewater

		HAV	HEV
Country/Area	**Year**	**Nested PCR**	**Semi-nested PCR**
Spain/Barcelona	1994–2002	31/54* (57.4%)	20/46* (43.5%)
Spain/Barcelona	2000–2004	5/18 (27.8%)	7/18 (38.9%)
Spain/Barcelona	2006–2008	1/32 (3.1%)	9/32 (28.1%)
Spain/Valencia	2007–2008	1/41 (2.4%)	13/41 (31.7%)

The results for nested and semi-nested assays are represented as positive samples/samples analysed (percentage of positives samples).
*The results shown were obtained from previous studies Pina *et al.* (2001) and Clemente-Casares *et al.* (2003), respectively.

Concentration of HAdV in wastewater samples

The level of human faecal contamination of the sampling sites was evaluated by determining the concentration of HAdV using QPCR. As expected, HAdV was detected in 100% of the analysed samples and the main value of concentration was 1.5×10^3 GC/ml of water tested. These values are frequently observed in urban sewage and are used as a marker of faecal contamination in contaminated water (Bofill-Mas *et al.* 2006).

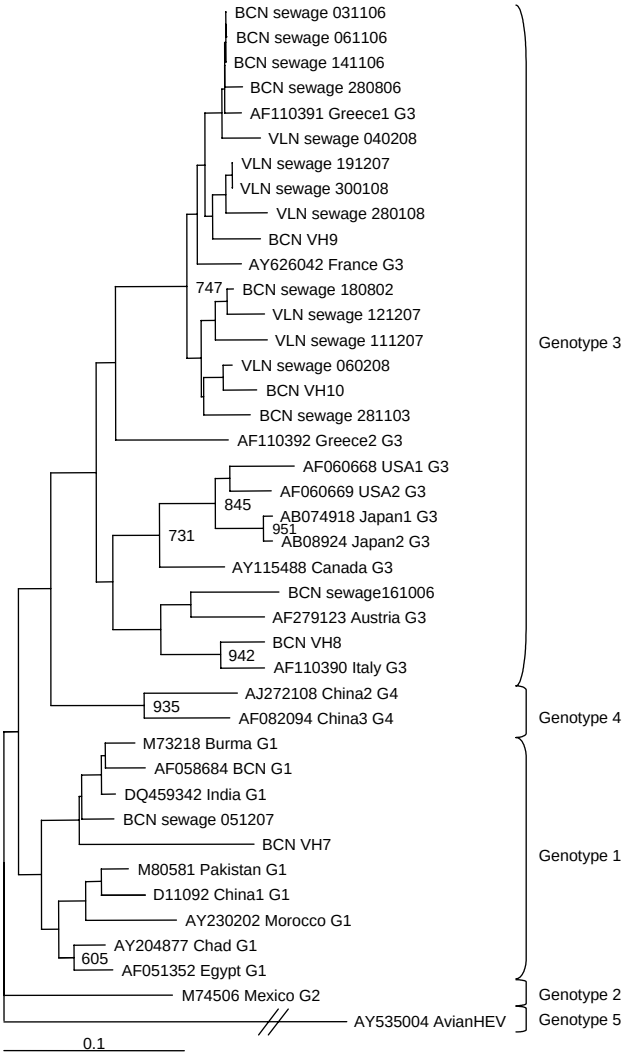

Figure 1 | Dendogram constructed by alignment of the 101 nucleotide sequences within the ORF2 region of HEV based on the neighbour-joining method. An avian HEV strain is included as outgroup. GenBank accession number and genotype (G) are reported. BCN (Barcelona) and VLN (Valencia) are the strains obtained in this study. Strains obtained from human sera are indicated as BCN VH.

Genetic characterization of the HAV and HEV strains

The sequences of HAV and HEV detected were compared with sequences described in previous studies in the same area and sequences available in databases. Five out of seven positive HAV sequences detected by amplification of the VP1/2A region (290 bp) were genotyped. The strains detected in Spain belonged to genotype IA and IB. Only two urban sewage samples were positive for HAV among the samples collected in Spain between 2006 and 2008, one from Barcelona, genotype IB, and one from Valencia, genotype IA.

Fifteen out of 29 positive samples for HEV were genotyped and the HEV strains detected were analysed by comparing the 101 bp sequence amplified in the ORF2 region. As expected, most of the HEV strains detected in samples from Barcelona and Valencia corresponded to genotype 3. However, in one sample from Barcelona, a genotype 1 HEV strain was identified. Three of the HEV strains identified from serum samples belonged to genotype 3, and one to genotype 1, the latter case corresponding to a person that had travelled to India during the previous month. Phylogenetic relationships between HEV strains isolated in Barcelona and Valencia were established by comparing the representative strains (Figure 1).

CONCLUSIONS

A previous study on HEV in the area around Barcelona (Clemente-Casares *et al.* 2003) showed a prevalence of positive samples of 43.5% (20/46) in urban sewage samples collected during 1994–2002. In the last years the prevalence appeared to be established at around 30% in the two study areas of Spain, with average concentrations of 3.2×10^1 GC/ml. These results demonstrate that HEV is circulating in the human population of this area and that urban sewage may be a source of contamination by this pathogen. Moreover, the information available on the high prevalence of HEV infections in swine (Meng *et al.* 1999; Pina *et al.* 2000) strongly suggests that HEV strains of swine origin transmitted through direct or indirect contact with infected pigs or through contaminated swine products or manure contribute to the circulation of HEV genotype 3 in the population.

The results of the present study show no detectable differences in the level of excretion of HAV in Barcelona, a place where all pre-adolescents have been vaccinated against HAV since 1999, compared to Valencia, where only risk groups are vaccinated. This observation strongly suggests that the reduction in HAV circulation does not occur in a single area of Spain, nor is this decrease related to a specific vaccination program. For HAV, 27.8% of sewage samples collected from 2000 to 2004 were positive.

This period could be considered a transition period and the results could be attributed to the ongoing improvements in sanitation. From the samples collected since 2006, HAV was detected in only one sample (3.1%) from Barcelona and in one sample (2.4%) from Valencia. Meanwhile, during this study, HAV was analysed in faecally contaminated water samples collected in north Egypt, an endemic area for this virus (Fix *et al.* 2006), which was present in a very high proportion of analysed samples (authors' unpublished results). Implementation of wastewater treatments and sewage treatment plants is a determinant factor in reducing faecally transmitted diseases in a population. From 1992 to 2008, the region of Catalonia experienced a significant increase in the number of sewage treatment plants (from 91 to 343) and in the total volume of wastewater depurated (from 991,892 to 2,786,871 m^3/day).

The results obtained on the prevalence of HEV showed a more stable excretion pattern in the population of NE Spain, with percentages of 28.1, 38.9 and 31.7% of positive urban sewage samples over the years studied after 2000 (Table 1). The concentration of HEV in positive urban sewage samples was lower than that of HAV, being 10^1 CG/ml and 10^2 CG/ml, respectively. The observed concentration of HAdV was much higher, which registered more than 10^3 CG/ml in all the samples analysed from Spain. This finding demonstrates the presence of faecal contamination in the samples studied and supports the applicability of human adenoviruses as indicators of human faecal contamination.

The studies of the amplified viral sequences showed the variability of HAV and HEV strains present in sewage. Although the sizes of the sequences were small, previous comparative studies have shown that the sequences analysed can be used for typification studies. In addition, the phylogenic trees produced in this study show equivalent topographies to those obtained when longer sequences were analysed (Pina *et al.* 2001; Clemente-Casares *et al.* 2003; Bofill-Mas *et al.* 2006). All the viral sequences obtained differed from the positive controls used in the experiments, thereby demonstrating the absence of cross contamination in the study. All the HEV strains identified were classified as genotype 3, except one sample which was in genotype 1. Furthermore, the genotyping of the viruses identified in clinical serum samples revealed the same results as for HEV, that is to say, most cases showed the presence of genotype 3, while one sample belonged to genotype 1, in this case in a patient who had recently travelled to India. The variability of HEV sequences detected in urban sewage samples in the studied area shows that diverse genotype 3 strains simultaneously circulate in the population. The sporadic detection of HEV genotype 1, considered typical of endemic regions and without animal reservoirs identified to date, indicates that HEV infections are well established in the human population in Europe and that a small proportion of genotype 1 strains also circulate in industrialized countries.

The results obtained in this study strongly suggest that HEV has replaced HAV as the most frequently detected hepatitis virus potentially transmitted through local faecally contaminated water or food in eastern Spain.

In industrialized countries such as the United States, a decline in the frequency of hepatitis A and in the mortality rate was observed in the past decade (serologic results and other approach) (Bell *et al.* 2005; Vogt *et al.* 2008). The study of the viruses present in urban sewage provides a useful approach to obtaining global information on their molecular epidemiology considering also subclinical infections in the population (Pina *et al.* 2001).

The dramatic reduction in the presence of HAV in sewage observed in eastern Spain could be most likely related to improvements in sanitation. However, these improvements have not had an equivalent effect on the circulation of HEV genotype 3 in the area. The continued circulation of this genotype would be maintained through animal hosts as HEV infections in swine represent an external source of HEV in the human population.

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REFERENCES

Balayan, M. S. 1997 Epidemiology of hepatitis E virus infection. *J. Viral Hepat.* **4**(3), 155–165.

Bell, B. P. 2002 Global epidemiology of hepatitis A: implications for control strategies. In Tenth International Symposium on Viral Hepatitis and Liver Disease, Atlanta, GA, USA, 9–13 April 2002, p. 9.

Bell, B. P., Kruszon-Moran, D., Shapiro, C. N., Lambert, S. B., McQuillan, G. M. & Margolis, H. S. 2005 Hepatitis A virus infection in the United States: Serologic results from the Third National Health and Nutrition Examination Survey. *Vaccine* **23**(50), 5798–5806.

Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-Manzano, J., Allard, A., Calvo, M. & Girones, R. 2006 Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. *Appl. Environ. Microbiol.* **72**(12), 7894–7896.

Boom, R., Sol, C. J. A., Salimans, M. M. M., Jansen, C. J., Wertheim-van Dillen, P. M. E. & Van der Noordaa, J. 1990 Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**(3), 495–503.

Buti, M., Domínguez, A., Plans, P., Jardí, R., Schaper, M., Espuñes, J., Cardeñosa, N., Rodríguez-Frías, F., Esteban, R., Plasència, A. & Salleras, L. 2006 Community-based seroepidemiological survey of hepatitis E virus infection in Catalonia, Spain. *Clin. Vaccine Immunol.* **13**(12), 1328–1332.

Clemente-Casares, P., Pina, S., Buti, M., Jardi, R., Martín, M., Bofill-Mas, S. & Girones, R. 2003 Hepatitis E virus epidemiology in industrialized countries. *Emerg. Infect. Dis.* **9**(4), 448–454.

Clemente-Casares, P., Rodriguez-Manzano, J. & Girones, R. 2009 Hepatitis E virus genotype 3 and sporadically also genotype 1 circulate in the population in Spain. *J. Water Health* **7**(4), 664–673.

Departament de Salut, Generalitat de Catalunya 2008 (available at http://www.gencat.cat/salut).

Direcció general de salut pública, conselleria de sanitat, Generalitat Valenciana 2008 (available at http://www.sp.san.gva.es/rvn/).

Emerson, S. U. & Purcell, R. H. 2004 Running like water–the omnipresence of hepatitis E. *N. Engl. J. Med.* **351**(23), 2367–2368.

Emerson, S. U., Anderson, D., Arankalle, A., Meng, X. J., Purdy, M., Schlauder, G. G. & Tsarev, S. A. 2005 Hepevirus. In *Virus Taxonomy, VIIIth Report of the ICTV* (ed. C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball), pp. 853–857. Elsevier Academic Press, London.

Erker, J. C., Desai, S. M. & Mushahwar, I. K. 1999 Rapid detection of Hepatitis E virus RNA by reverse transcription-polymerase chain reaction using universal oligonucleotide primers. *J. Virol. Methods* **81**(1–2), 109–113.

Fix, A. D., Abdel-Hamid, M., Purcell, R. H., Shehata, M. H., Abdel-Aziz, F., Mikhail, N., el Sebai, H., Nafeh, M., Habib, M., Arthur, R. R., Emerson, S. U. & Strickland, G. T. 2006 Prevalence of antibodies to hepatitis E in two rural Egyptian communities. *Am. J. Trop. Med. Hyg.* **62**(4), 519–523.

Jacobsen, K. H. & Koopman, J. S. 2004 Declining hepatitis A seroprevalence: a global review and analysis. *Epidemiol. Infect.* **132**(6), 1005–1022.

Jothikumar, N., Cromeans, T. L., Hill, V. R., Lu, X., Sobsey, M. D. & Erdman, D. D. 2005*a* Quantitative real-time PCR assays for detection of human adenoviruses and identification of serotypes 40 and 41. *Appl. Environ. Microbiol.* **71**(6), 3131–3136.

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

Jothikumar, N., Cromeans, T. L., Robertson, B. H., Meng, X. J. & Hill, V. R. 2006 A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *J. Virol. Methods* **131**(1), 65–71.

Legrand-Abravanel, F., Mansuy, J. M., Dubois, M., Kamar, N., Peron, J. M., Rostaing, L. & Izopet, J. 2009 Hepatitis E virus genotype 3 diversity, France. *Emerg. Infect. Dis.* **15**(1), 110–114.

Martín, M., Segalés, J., Huang, F. F., Guenette, D. K., Mateu, E., de Deus, N. & Meng, X. J. 2007 Association of hepatitis E virus (HEV) and postweaning multisystemic wasting syndrome (PMWS) with lesions of hepatitis in pigs. *Vet. Microbiol.* **122**(1–2), 16–24.

Meng, X. J., Purcell, R. H., Halbur, P. G., Lehman, J. R., Webb, D. M., Tsareva, T. S., Haynes, J. S., Thacker, B. J. & Emerson, S. U. 1997 A novel virus in swine is closely related to the human hepatitis E virus. *Proc. Natl Acad. Sci. USA* **94**(18), 9860–9865.

Meng, X. J., Dea, S., Engle, R. E., Friendship, R., Lyoo, Y. S., Sirinarumitr, T., Urairong, K., Wang, D., Wong, D., Yoo, D., Zhang, Y., Purcell, R. H. & Emerson, S. U. 1999 Prevalence of antibodies to the hepatitis E virus in pigs from countries where hepatitis E is common or is rare in the human population. *J. Med. Virol.* **59**(3), 297–302.

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.

Orrù, G., Masia, G., Orrù, G., Romanò, L., Piras, V. & Coppola, R. C. 2004 Detection and quantitation of hepatitis E virus in human faeces by real-time quantitative PCR. *J. Virol. Methods* **118**(2), 77–82.

Paul, D. A., Knigge, M. F., Ritter, A., Gutierrez, R., Pilot-Matias, T., Chau, K. H. & Dawson, G. J. 1994 Determination of hepatitis

E virus seroprevalence by using recombinant fusion proteins and synthetic peptides. *Infect. Dis.* **169**(4), 801–806.

Pina, S., Jofre, J., Emerson, S. U., Purcell, R. H. & Girones, R. 1998 Characterization of a strain of infectious hepatitis E virus isolated from sewage in an area where hepatitis E is not endemic. *Appl. Environ. Microbiol.* **64**(11), 4485–4488.

Pina, S., Buti, M., Cotrina, M., Piella, J. & Girones, R. 2000 HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. *J. Hepatol.* **33**(5), 826–833.

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

Puig, M., Jofre, J., Lucena, F., Allard, A., Wadell, G. & Girones, R. 1994 Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. *Appl. Environ. Microbiol.* **60**(8), 2963–2970.

Purcell, R. H. & Emerson, S. U. 2008 Hepatitis E: an emerging awareness of an old disease. *J. Hepatol.* **48**(3), 494–503.

Siegl, G., Weitz, M. & Kronauer, G. 1984 Stability of hepatitis A virus. *Intervirology* **22**(4), 218–226.

US Food and Drug Administration 2001 FDA Talk Paper (available at http://www.fda.gov/bbs/topics/ANSWERS/2001/ANS01084.html).

Vogt, T. M., Wise, M. E., Bell, B. P. & Finelli, L. 2008 Declining hepatitis A mortality in the United States during the era of hepatitis A vaccination. *J. Infect. Dis.* **197**(9), 1282–1288.

Yarbough, P. O. 1999 Hepatitis E virus. Advances in HEV biology and HEV vaccine approaches. *Intervirology* **42**(2–3), 179–184.

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