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Human Viruses in Water
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Chapter 3

# Enteric Hepatitis Viruses

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## Background

The term "jaundice" was used as early as in the ancient Greece when Hippocrates described an illness probably corresponding to a viral hepatitis. However, it was not until the beginning of the twentieth century when a form of hepatitis was associated to an infectious disease occurring in epidemics and the term "infectious hepatitis" was established. Later on in the early 1940s two separate entities were defined: "infectious" and "serum" hepatitis, and from 1965 to nowadays the different etiological agents of viral hepatitis have been identified. Although all viral hepatitis are infectious the aforementioned terms refer to the mode of transmission, corresponding the "infectious" entity to those hepatitis transmitted through the fecal-oral route and the "serum" hepatitis to those transmitted parenterally. Thus, the infectious or enteric hepatitis include two types: hepatitis A and hepatitis E, which will be reviewed here.

AU :1

## Hepatitis A

### *Natural course and epidemiology of hepatitis A*

Hepatitis A infection may develop asymptomatically. This type of subclinical infection is most common among young children (under 5), while in older children

and in the adulthood the infection usually proceeds with symptoms (Previsani et al., 2004). In this latter case, the clinical course of hepatitis A is indistinguishable from that of other types of acute viral hepatitis. The clinical case definition for hepatitis A is an acute illness with moderate onset of symptoms (fever, malaise, anorexia, nausea, abdominal discomfort, dark urine) and jaundice, and elevated serum bilirubin and aminotransferases levels later on. The incubation period of hepatitis A ranges from 15 to 50 days and clinical illness usually does not last longer than 2 months, although 10–15% of patients have prolonged or relapsing signs and symptoms for up to 6 months (Sjogren et al., 1987; Glikson et al., 1992). In fact, with the advent of new highly sensitive techniques even in normal clinical courses a high and long lasting viremia has been detected (Costafreda et al., 2006), with the peak (up to $10^7$ genome copies/ml of sera) occurring at 2 weeks after the onset of symptoms and lasting up to an average of 6 weeks after the start of symptoms (Bower et al., 2000; Costafreda et al., 2006). However, there is no evidence of chronicity of the infection. Hepatitis A infection may occasionally produce fulminant hepatitis, mainly among patients with underlying chronic liver diseases (Akriviadis and Redeker, 1989; Previsani et al., 2004).

The distribution patterns of hepatitis A in different geographical areas of the world are closely related to their socioeconomic development (Gust, 1992; Hollinger and Emerson, 2001; Previsani et al., 2004). The endemicity is low in developed regions and high in underdeveloped countries. The epidemiological pattern has important implications on the average age of exposure and hence, as above stated, on the severity of the clinical disease. Since hepatitis A infection induces a life-long immunity (Hollinger and Emerson, 2001), severe infections among adults are rare in highly endemic regions where most children are infected early in life. In contrast, in low endemic areas the disease occurs mostly in adulthood, mainly as a consequence of traveling to endemic regions, or as food or waterborne outbreaks, and hence the likelihood of developing severe symptomatic illness is high. An epidemiological shift, from intermediate to low prevalence, has been noticed in recent decades in many countries, particularly in southern Europe, including Spain, Italy and Greece (Germinario and Lopalco, 1999; Salleras, 1999; Van Damme et al., 1999). Consequently, the Mediterranean basin as a whole should no longer be considered as an endemic area (Previsani et al., 2004; Pintó et al., 2006). Additionally, some other countries from eastern Europe (Cinciara, 2000; Tallo et al., 2003) have also described significant declines in the incidence of hepatitis A. AU :2

### *General features of hepatitis A virus (HAV)*

The etiological agent of hepatitis A is the hepatitis A virus (HAV) which belongs to genus *Hepatovirus* within family *Picornaviridae*, and as such it consists of a non-envoloped icosaedral capsid of around 30 nm in diameter containing a positive ssRNA genomic molecule of 7.5 Kb (Fauquet et al., 2005). The genome contains a single open reading frame (ORF) encoding a polyprotein of around 2225 amino acids (aa) preceded by a 5′ non-coding region (5′NCR) that makes around 10% of

the total genome, and followed by a much shorter 3′NCR that contains a poly(A) tract (Baroudy et al., 1985; Cohen et al., 1987). This genome is uncapped but AU :3
covalently linked to a small viral protein (VPg) (Weitz et al., 1986). The singly translated polyprotein is subsequently cleaved into 11 proteins through a cascade of proteolytic events brought about mainly by the viral 3C protease (Schultheiss et al., 1994; Schultheiss et al., 1995). However, although the general genomic organization and the expression pattern of HAV are very similar to those of most picornaviruses (Hollinger and Emerson, 2001; Agol, 2002), many differences exist which deserve a special attention.

### *What makes hepatitis A virus such a special picornavirus*

The genetic distance between the genus *Hepatovirus* and the other genera of the family reflects not merely a difference in the nucleotide and amino acid composition but a difference in the molecular and biological characteristics of HAV. From the genomic and proteomic points of view, several interrelated key issues must be brought up. First of all, the structure of the 5′NCR and its internal ribosome entry site (IRES). It is likely that picornavirus IRES has evolved by gradual addition of domains and elements that improved its function in ribosome recruitment or otherwise conferred regulation to the process of viral protein synthesis in a specific cell environment (Ehrenfeld and Teterina, 2002). The HAV IRES is unique among picornaviruses and constitutes the type III model (Braun et al., 1994; Ehrenfeld and AU :4
Teterina, 2002), which shows a very low efficiency in directing translation (Whetter et al., 1994). Second, HAV encodes only a protease, 3C, while other picornavirus code for additional proteases such as the L protease, in genus *Aphtovirus*, or the 2A protease in *Enterovirus* and *Rhinovirus* genera (Leong et al., 2002). L and 2A proteases, when present, play a crucial role in the primary cleavages of the viral polyprotein while in those genera lacking these proteases, such as *Hepatovirus* and *Paraechovirus*, both primary and secondary cleavages are conducted by the 3C protease. But what is most important is that these additional proteases are involved in the cellular protein shutoff induction (Leong et al., 2002). Since picornaviruses utilize a mechanism of translation that is cap-independent and IRES-dependent, the inhibition of non-essential cap-dependent cellular translation could be advantageous to the virus. In doing so, the cellular translation machinery is utilized almost exclusively for the production of viral proteins (Kuechler et al., 2002). An early event preceding the shutoff of host cell protein synthesis is the cleavage of the cellular translation initiation factor eIF4G, and evidence exists supporting that the enzymes responsible of such a cleavage are 2A and L proteases in enteroviruses and rhinoviruses, and aphtoviruses, respectively (Kuechler et al., 2002). An immediate consequence of the lack of any of these proteolytic activities in HAV is its incapacity to induce cellular shutoff which otherwise is directly related with its requirement for an intact uncleaved eIF4G factor for the formation of the initiation of translation complex (Borman et al., 1997; Jackson, 2002). An intriguing evolutionary question remains to be solved regarding the selection of such an

inefficient IRES in HAV. An explanation has been suggested which accounts for the constraints caused by the need to accommodate the dual functions of translation and replication in adjacent regions of the 5′NCR, generating inadvertent consequences for either function (Ehrenfeld and Teterina, 2002). What has been described up to now denotes that HAV must inefficiently compete for the cellular translational machinery and thus it presents a unique translation strategy. This points out to the third difference between HAV and other picornavirus members: the codon usage. HAV presents a higher codon usage bias compared to other members of its family, which conveys in the adaptation to use abundant and rare codons (Sánchez et al., 2003b). In fact, 14 aa families contain rare codons, defined in terms of their frequencies, making a total of 22 used rare codons. But what is more surprising is that the HAV codon usage has evolved to be complementary to that of human cells, never adopting as abundant codons those abundant for the host cell, and even in some instances using these latter as rare codons. This disparity, unique to HAV, has been interpreted as a subtle strategy to avoid, as much as possible, competition for the cellular tRNAs in the absence of a precise mechanism of inducing shutoff of cellular protein synthesis (Sánchez et al., 2003b). As stated before, a consequence of this special codon bias is an increase in the number of rare codons used by HAV. Overall this increment is the result of the addition to the cellular rare codons, also used as rare by the virus, of those most abundant cellular codons that being unavailable for the virus are used at low frequencies. Altogether, the HAV codon usage may contribute to its slow replication and to its low yields. It has been largely documented, (Robinson et al., 1984; Sørensen et al., 1989; Chou and Lakatos, 2004) the role of rare codons in the control of translation AU :5 speed, in the sense that clusters of rare codons would induce a transient stop of the translational complex in order to seek for a suitable tRNA present at a very low concentration among the pool of tRNAs. A function of these ribosome stallings has been suggested to be the assurance of the proper folding of the nascent protein (Adzhubei et al., 1996; Gavrilin et al., 2000; Evans et al., 2005). Such a function may be postulated for HAV, where highly conserved clusters of rare codons strategically located at the carboxi-ends of the structured elements have been reported (Sánchez et al., 2003b). A certain contribution of the codon usage to the low variability of the HAV capsid has been proposed taking into account that 15% of its surface residues are encoded by such functional rare codons (Sánchez et al., 2003b). This low capsid variability indeed correlates with a very low antigenic variability: a single serotype exists, this being another striking difference with other picornaviruses. The low capsid variability should rely on negative selection acting against potential newly arising proteins, since the viral population replicates as a quasispecies (Sánchez et al., 2003a). The quasispecies analysis revealed a dynamics of mutation selection at and around the rare codons, confirming a seminal role of the codon usage on HAV evolution (Sánchez et al., 2003a).

Other important differences exist between HAV and other picornaviruses at the morphogenetic/structural level. The role of both ends (amino-VP4 and carboxi-2A) of the capsid polyprotein in the virion assembly is still controversial (Probst et al.,

1999), and while there is no agreement on the requirement of VP4 for the maturation of pentamers into capsids (Probst et al.,1999; Martin and Lemon, 2006), a complete consensus exists on the necessity of 2A for pentamer formation (Probst et al., 1999; Martin and Lemon, 2006). The ulterior removal of 2A in the mature virion must be performed by a host cell protease (Graff et al., 1999; Martin et al., 1999), although the mature 2A protein has never been identified directly in infected cells. AU :6

The X-ray crystallographic structure has not yet been solved, due to the low viral yields obtained by *in vitro* replication. However, recent 3D images of HAV produced by cryoelectron microscopy (Holland Cheng, unpublished results) have revealed important data being the most intriguing the lack of a well-defined canyon around the fivefold axis of symmetry. The pit region of many picornaviruses contains the receptor binding residues (Rieder and Wimmer, 2002) playing, thus, an important biological role. A human HAV receptor (huhavr-1) has been identified (Feigelstock et al., 1998), which contains both an amino terminal Ig-like domain followed by a mucin-like domain (Silberstein et al., 2003). Huhavr-1 has been detected in several human tissues, including the liver. Alternatively the asialoglycoprotein receptor, to which IgA binds to, has been described to enable HAV internalization provided that the virus is complexed with such immunoglobulin (Dotzauer et al., 2000). However, whichever is the receptor, the capsid region involved in such an interaction remains to be elucidated. In contrast, the capsid region interacting with the glycophorin A of the human erythrocytes is indeed located around the putative pit area (Sánchez et al., 2004a,b). The capsid structure, however, is such that it tolerates this interaction only to occur at acid conditions, being impaired at neutral biological conditions. Erythrocyte glycoproteins may function as decoy receptors attracting pathogens to the erythrocyte and keeping them away from target tissues (Gagneux and Varki, 1999), and hence the actual capsid conformation that allows escaping from erythrocyte attachment may constitute an advantage for a viremic infectious agent whose target organ is the liver. In fact, pathogenesis is in part determined by the spread of the virus to the target tissues (Rieder and Wimmer, 2002). In this context, key factors for the viral biological cycle and infection outcome are a high stability to the acid pH of the stomach during the entry phase, a safe viremic phase, and resistance to the action of detergents, particularly biliary salts, during the exit phase. This extreme resistant phenotype of HAV explains its high persistence in the environment (Abad et al., 1994a,b) and its transmission by contaminated foods and drinking water (Reid and Robinson, 1987; Rosemblum, 1990; Bosch et al., 1991; Dentinger et al., 2001; Sánchez et al., 2002), which probably are the result of a highly cohesive capsid conformation mediated through a very accurate folding.

### *Hepatitis A transmission*

Hepatitis A is shed in the feces of infected patients. The viral concentration in such stools is highest (up to $10^{11}$ genome copies/g of feces) after two weeks of the onset

of symptoms and lasts at least four more weeks (Costafreda et al., 2006). HAV infection is mainly propagated via the fecal-oral route as the person-to-person contact is the most common mode of transmission (Mast and Alter, 1993). In fact HAV survival in contaminated fomites, such as sanitary paper, sanitary tile and latex gloves, is very long (Abad et al., 1994a). In consequence, given the high excretion level of HAV, transmission of the infection is facilitated when poor sanitary conditions occur. Nevertheless, transmission through the parental route may occasionally occur (Noble et al., 1984; Sheretz et al., 2005).

AU :7

*Contaminated water and food as critical elements in the route of transmission*

Viruses present in the stool of infected patients are discharged into sewage which ultimately may contaminate surface waters and seawater, and consequently be acquired and concentrated by shellfish growing in these waters, or contaminate the vegetables irrigated with the polluted waters. While in approximately 40% of the reported cases of hepatitis A the source of infection cannot be identified, waterborne and foodborne outbreaks of the disease have been reported. Within this latter category, shellfish grown and harvested from waters receiving urban contaminants is a cause of large outbreaks of infectious hepatitis (Halliday et al., 1991; Sánchez et al., 2002). Additionally, large outbreaks associated with the consumption of berry fruits (Reid and Robinson, 1987) and vegetables (Rosemblum et al., 1990; Dentinger et al., 2001) irrigated with contaminated waters may occur. Waterborne outbreaks are less common since the introduction of drinking water treatments. However, reports exist when these measures fail and outbreaks of hepatitis A occur (Bosch et al., 1991).

## *Detection and quantification of hepatitis A virus*

As above stated, HAV may contaminate different types of environmental samples including sewage, surface waters and seawater, and ultimately drinking water and foodstuffs. Thanks to its high stability under very different conditions (Abad et al., 1994b; Bosch et al., 1994), HAV can persist long enough in these edible samples to be transmitted through their ingestion. Thus, although not compulsory, the screening of HAV presence is advisable in specific samples, at least when suspicion of contamination exists.

Cell culture propagation of wild-type strains of HAV is a complex and tedious task, which requires virus adaptation before its effective growth, and even in this case, the virus usually establishes persistent infections resulting in low virus yields (Flehmig, 1980; Daemer et al., 1981; Wang et al., 1986). Thus, infectivity is not nowadays a useful method for primary HAV detection. Alternatively, immunological and, particularly, molecular techniques should be used.

*Molecular methods versus immunological methods: a clear choice in water virology*

Although fecal excretion of HAV is high, environmental samples usually contain low viral numbers due mainly to the effect of dilution but also to some extent of virus inactivation. Most of the immunological techniques used in clinical diagnosis are aimed at the detection of those antibodies raised upon virus infection, mainly IgM and IgG anti-HAV (Nainan et al., 2006), while antigen detection is uncommon. Additionally, to the lack of immunological kits for antigen detection, their sensitivity would not be high enough to be employed in scenarios of low environmental virus concentration. All this calls for the development of highly sensitive methods for HAV detection, such as those based on nucleic acids amplification. The adoption of these techniques requires selection of the most adequate amplification target. The target region should be highly conserved to increase the chance of detection and with an appropriate structure and length to allow the required sensitivity. Immunological evidence determines the existence of a single serotype of HAV (Lemon and Binn, 1983), although genomic analysis of the virus allows the differentiation of six genotypes (Robertson et al., 1992; Costa-Mattioli et al., 2003). However, all six genotypes are very closely related in the 5′NCR, which is the most conserved region of the genome due to its functional structure in the processes of translation and replication, as above stated, and with a maximum nucleotide divergence of less than 5%. Consequently, the 5′NCR is the region of choice for the design of broad spectrum molecular techniques.

*Genetic diversity*

In spite of the low antigenic variability of HAV, a degree of nucleotide variability similar to that of other picornavirus in the capsid coding region has been described (Sánchez et al., 2003b). This is explained by a very low number of non-synonymous mutations per non-synonymous site and a number of synonymous mutations per synonymous site similar to that of other picornaviruses. This genetic diversity allows the differentiation of HAV into several genotypes and subgenotypes. Different genomic regions have been used to differentiate the genotypes, including the carboxi-terminus of VP3, the amino-terminus of VP1, the VP1 × 2A junction, the region spanning the carboxi-end of VP1 till the amino-terminus of 2B (VP1/P2B), and finally the entire VP1 region (see the review of Nainan et al., 2006). However, partial genomic sequences never will guarantee the reliability of the complete VP1/2A region. As a matter of fact, the identification of some HAV antigenic variants affecting residues not included in the genotyping regions (Costa-Mattioli et al., 2002a; Sánchez et al., 2002; Gabrieli et al., 2004) could have been elusive in such circumstances. This is the reason why the use of long genomic regions covering at least the entire VP1 including its 2A junction, has recently been recommended (Costa-Mattioli et al., 2002a; European HAV Network, unpublished results) for a more broad molecular typing of HAV. However, the VP1 × 2A junction is still the genomic region most in use worldwide (Robertson et al., 1992). In

this region, seven genotypes were initially defined, whose genetic distance was >15% nucleotide variation. After refining this classification through the addition of more sequences, only six genotypes exist at the present time (Costa-Mattioli et al., 2002a; Lu et al., 2004). Three out of these six genotypes (I, II and III) are of human origin while the other (IV, V and VI) are of simian origin. Genotypes I and II contain subgenotypes (Ia, Ib, IIa and IIb) defined by a nucleotide divergence of 7–7.5%.

Whether the objective is the general broad spectrum detection of HAV or the typing of the isolated strains will determine the use of 5′NCR or VP1 × 2A targets (Sánchez et al., 2004a,b) or, alternatively, other genotype targets.

*The advent of real-time standardized quantitative techniques for the accurate estimation of the HAV titer*

Many molecular techniques have been used throughout the years for HAV detection, including hybridization (Bosch et al., 1991; Zhou et al., 1991) and amplification techniques, essentially RT-PCR, usually combined with confirmation tests such as the southern blotting (Calder et al., 2003; Sánchez et al., 2004a,b ), restriction fragment length polimorfism (Goswami et al., 1997) or sequencing (Sánchez et al., 2002). Recently, new approaches have been developed which enable not only the detection but the quantification of the genome copy numbers in a real-time scale. These techniques basically include two different alternatives: the real-time RT-PCR and the nucleic acid sequence based amplification (NASBA). Both techniques take advantage of the use of a combination of enzymes for the amplification of RNA and a reliable method to quantify the final amplified product, usually based on the use of fluorescent probes. In the real-time RT-PCR, the gold standard is the combination of the reverse trascriptase (RT) and the *Taq* polymerase to transform the target ssRNA into the final dsDNA, while the NASBA is again the RT this time with the T7 RNA polymerase and a primer including the T7 promoter that allows the synthesis of great amounts of the final RNA product. Both combinations show a high intrinsic amplification power and although it has been claimed that the NASBA technique allows an even higher amplification than the real-time RT-PCR, more descriptions exist on the use of this latter technique (Costa-Mattioli et al., 2002b; Jothikumar et al., 2005; Costafreda et al., 2006) than with the NASBA (Jean et al., 2001; Abd El Galil et al., 2005) for the quantification of HAV.

Two key issues have to be solved when real-time quantification techniques are developed. Finding those optimal conditions for each particular target rather than using universal settings. It has been claimed that the use of one-step RT-PCR formats reduces the HAV detection sensitivity by up to 1 log unit (Nainan et al., 2006) in comparison to two-step formats. However, when optimum conditions are established for each format, the same level of sensitivity is achieved irrespective of the use of one-step or two-step formats (Costafreda et al., 2006). Not only is the establishment of optimal conditions crucial in the success of a highly sensitive

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quantification technique, but also standardization of the procedures. Detection of viral RNA by molecular amplification procedures involves several essential steps, the viral RNA extraction and the reverse transcription being the most critical ones. Since no universal reliable nucleic acid extraction method exists, the best option is to control the efficiency of extraction for a particular matrix. The addition of a known concentration of an external virus control to the samples to be quantified has been proposed to assess the efficiency of RNA extraction of enteric viruses in different matrices. Vaccinal poliovirus type 2 (Nishida et al., 2003) and vaccinal Mengo virus (Costafreda et al., 2006) have been proposed as such controls. However, poliovirus presents the inconvenience of being itself a potential contaminant of samples and additionally its use in the context of the poliomyelitis eradication era (see chapter by Hovi et al.) is not advisable. The control of the reverse transcription reaction is more widely performed and most of the real-time techniques described for the quantification of HAV include an RNA standard internal control (Costa-Mattioli et al., 2002b; Jothikumar et al., 2005; Costafreda et al., 2006). Usually, these controls are synthesized by *in vitro* transcription of cloned cDNAs or T7 promoter-containing amplimers corresponding exactly to the targets, and are added to each test tube at known amounts in order to follow up the efficiency of the reaction. Applying these control measures, a fine estimation of the number of HAV genome copies per gram of shellfish was performed for the first time in several clam stocks associated with an outbreak of hepatitis A, and ranged from $1 \times 10^3$ to $1 \times 10^5$ (Costafreda et al., 2006). These determinations have provided the means for a risk-assessment study linking the level of shellfish contamination and the attack rate of infection (Costafreda et al., unpublished results).

### *Molecular epidemiology of hepatitis A: the water environment as an overview of the circulating viruses*

Environmental surveillance, mostly sewage monitoring, has been used to assess the circulation of different human enteric viruses, notably rotaviruses (Villena et al., 2003; van Zyl et al., 2006), wild-type poliovirus (Deshpande et al., 2003; El Bassioni et al., 2003) and also HAV (Pintó et al., 2006) as well as animal enteric viruses (Jimènez-Clavero et al., 2003, 2005a). The application of molecular typing techniques in this kind of samples allows the molecular and phylogenetic analysis required for the traceability of strains, i.e. in the context of the poliovirus eradication program (see chapter by Hovi et al.). The molecular characterization of viral strains isolated in sewage, i.e. rotavirus, might be very useful in the policy for the design for future vaccines applicable to countries where the clinical surveillance does not cover all the population.

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Environmental molecular epidemiology as such should rely not only on the molecular characterization of the isolated strains but also on the epidemiological data regularly collected. When both disciplines are applied together interesting conclusions might be drawn. In the particular case of HAV, a study relating a vaccination campaign, the number of clinical cases and the occurrence of viruses in

sewage during a period of 5 years in Barcelona city has been reported (Pintó et al., 2006). Attack rates per 100,000 inhabitants of 9.1, 6.2, 3.3, 1.7 and 8.0 were estimated during the years 1998–2002. While the progressive decline could be clearly associated to a vaccine administration the final increase was attributed to the huge immigration flow, from North Africa, South America and East Asia that the city received (nearly 10% of the total population). The vaccination campaign was, and is, dedicated to children 12 years old, being those $<12$ still susceptible to the infection. Immigrant children may act as potential carriers of the infection, and in fact many of the observed cases were school-related, and mostly coincided with the virus incubation period elapsed after the return from the school holiday, and after the immigrant population have returned from visits to their countries of origin. Sewage surveillance data taught us that the similar high attack rates seen at the beginning and final years of the study responded to a different infection pattern. While many asymptomatic cases might occur at the beginning with high levels of virus excreted and with many positive isolations in sewage, at the end only small outbreaks among the non-vaccinated population arose without the massive involvement of asymptomatic carriers and consequently without positivity in sewage. In conclusion, the environmental study reflected that in spite of the increase in clinical cases in the last year of study, the vaccination program was anyway working well.

Finally, it should be stated that molecular environmental surveillance still presents some intrinsic problems, genotyping being one of them. Even when using a short fragment such as the VP1 $\times$ 2A region, HAV genotyping is a hard task due to the low concentration of viruses occurring in sewage. Using this genotyping target, a threshold of $10^5$ genomes/ml has been estimated. In consequence, only those genotypes, more prevalent in the population, are likely to be detected. However in areas endemic for HAV, such as Egypt (Pintó et al., 2006), environmental surveillance is a powerful tool to complement the clinical epidemiology data, since detection and genotyping as well are possible. In fact, as above stated, this approach has been already implemented in Egypt in the context of the polio eradication program (El Bassioni et al., 2003).

### *Prevention of hepatitis A*

Inactivated HAV vaccines are available since the early 1990s and provide long-lasting immunity against hepatitis A infection (Bell and Feinstone, 2004). The immunity is largely related to the induction of high titers of specific antibodies. Thanks to the existence of a single serotype of HAV, these vaccines are of high efficacy. These vaccines consist of viruses grown in cell culture, purified, inactivated with formalin and adsorbed to an aluminum hydroxide adjuvant, making their economic cost quite high. This is the reason why many discrepancies already exist on their universal use in massive vaccination campaigns. Countries with previous intermediate endemicity of HAV such as Israel or some autonomous communities of Spain such as Catalonia, or some States of United States have performed studies

on the impact of child vaccination on the overall incidence of hepatitis A concluding that the immunization is medically (Salleras, 1999; Shouval, 1999; Wasley et al., 2005) and economically (Dagan et al., 2005; http://www.gencat.net/salut/portal/cat/nen.htm; http://http://www.cdc.gov/nip/publications/VIS/vis-hep-a.pdf) justified. In contrast, other countries in a similar situation such as Italy do not recommend at present the implementation of such a measure in terms of cost-benefits (Franco and Vitiello, 2003). In this context is quite evident that high endemic countries that usually have low economic incomes do not regard the vaccination against hepatitis A as a primary policy (Teppakdee et al., 2002).

Although several attenuated vaccine candidates have also been attempted, due to the successful use of inactivated vaccines, its development is hardly plausible.

As a general rule, in low and intermediate endemic regions, where paradoxically the severity of the disease is high, vaccination against hepatitis A should be recommended in high-risk groups, including travelers to high endemic areas, men having sex with men, drug users and patients receiving blood products. In addition, the inclusion of hepatitis A vaccines in mass vaccination programs in those countries receiving high numbers of immigrants from endemic countries is particularly advisable. However, and bearing in mind the quasispecies replication pattern of HAV (Sánchez et al., 2003a) that could lead in populations with continued exposure to the virus to the selection of new antigenic variants escaping immune protection, mass vaccination programs in highly endemic areas is controversial.

## Hepatitis E

### *Natural course and epidemiology of hepatitis E*

Hepatitis E, previously known as enterically transmitted non-A, non-B hepatitis, is an infection with clinical and epidemiological features of acute hepatitis. The clinical presentation of hepatitis E is basically similar to that of hepatitis A, but cholestatic jaundice is more common. The clinical course of the infection was first addressed in a human volunteer that ingested a clarified stool preparation from an infected patient (Balayan et al., 1983). The incubation period averages 40 days, with a range between 15 and 60 days. Amino alanine transferase elevation occurs during 30–120 days after infection, and fecal excretion of the virus begins around 1 week before onset of illness and continues for, at least, 2–3 weeks thereafter (Skidmore, 2002). The ecteric phase of the infection is characterized by a flu-like prodrome with epigastric pain, vomiting, fever and discoloration of the urine, but jaundice patients display yellowish skin, scleral icterus, dark urine, and light tan-colored feces; however most infections are asymptomatic and no evidence of chronic disease has been observed (Purcell and Emerson, 2001; Smith, 2001). As a result of viral replication in the liver, the hepatitis E virus (HEV) is found in the bile in large quantities, reaching the intestines by the bile duct and being subsequently shed in the feces. Asymptomatic infected individuals may shed virus and become reservoirs of the virus between epidemics and, therefore, contribute to sporadic

infection by person-to-person transmission or by contaminating water and food. In endemic regions, the overall attack rate was estimated to be around 2.5% in adults and around 1.2% in children (Vishwanathan, 1957). The case-fatality rate is usually low (0.2–3%), but in pregnant women during the third trimester of gestation it can be as high as 15–25%, primarily due to fulminant hepatic failure (Purcell and Emerson, 2001; Smith, 2001; Skidmore, 2002).

The significance of the host immune response in the pathogenesis of cell damage is not fully understood. Anti-HEV IgM appears at the time of the onset of symptoms and remains detectable for to 2–3 months. Anti-HEV IgG is detectable shortly after IgM detection, increases during the acute phase and may be present in serum for years after the initial infection (Clayson et al., 1995b).

There is no specific treatment for hepatitis E. Passive immunization with convalescent sera has been accomplished in animal models (Tsarev et al., 1994) but in humans administration of immune globulin obtained from inhabitants of HEV endemic regions was unsuccessful (Khuroo and Dar, 1992); however, it should be noted that this study used unselected plasma and that, even in endemic regions, anti-HEV prevalence and titers are low. Therefore, it cannot be ruled out that the use of selected anti-HEV batches of immunoglobulins with high titers may be useful, particularly for pregnant women and/or during epidemics.

HEV is transmitted primarily by contaminated water, and causes frequent epidemics in areas with inadequate water supplies and poor sanitary conditions (Purcell and Emerson, 2001), being the principal cause of acute, sporadic hepatitis in adults in many areas of Asia, Middle East and Northern Africa (Emerson and Purcell, 2003; Schlauder, 2004). Recently, an increase in the number of cases in regions considered as non-endemic for hepatitis E has been reported (Smith, 2001; Worm et al., 2002a).

Since the main route of HEV transmission is feco-oral, most epidemics can be linked to waterborne outbreaks, particularly in developing countries with warm weather, high population density and poor sanitary conditions. The first documented outbreak of HEV occurred in India in 1955–1956 (Vishwanathan, 1957). The origin of the outbreak, which was initially attributed to hepatitis A and later on confirmed to be hepatitis E, was the contamination by sewage, from 1 to 6 weeks prior to the epidemic, of Jumna River, the source of water for the treatment plant. Alum and chlorine treatment prevented bacterial infections, but 30,000 cases of hepatitis occurred among the population (Wong et al., 1980). One of the highest epidemic areas is China, where al least 11 epidemic outbreaks have been reported to date. The largest one occurred in 1986–1988, with more than 119,000 cases that resulted in more than 700 deaths. Until recently most cases reported in developed countries were attributed to travel to endemic areas, however, as aforementioned, there is an increase in the number of cases of infected patients that had never been abroad (Smith, 2001; Worm et al., 2002a).

In addition, a zoonotic potential for the virus was suggested after detection of HEV infection in wild and domestic animals, and later on confirmed in people who ate HEV infected uncooked deer meat (Meng, 2003; Goens and Purdue, 2004). The

risk of a zoonotic spread of the virus, its detection in non-endemic areas, and the continuous occurrence of outbreaks in endemic regions have boosted the interest in the understanding of the biology and life cycle of the virus, and in the improvement of diagnostic tools able to detect the pathogen in polluted waters and other environmental samples.

*Seroprevalence*

The overall anti-HEV prevalence reported in endemic countries is quite variable, but lower than expected, 3–27% (Purcell 1994; Purcell and Emerson, 2001; Worm et al., 2002b). In contrast to other enteric viruses such as poliovirus or HAV, the prevalence of anti-HEV IgG is lower in children than in adults (Arankalle et al., 1995, Meng, 2002). A possible explanation for this could be that HEV immunity acquired with subclinical infection during childhood wanes with time. In non-endemic areas with good sanitary conditions and control of water supplies, there is a low but constant increase in the number of HEV sporadic cases non-related to travel, and the anti-HEV antibody prevalence among the healthy population is relatively high, even higher than that reported in endemic areas (Meng et al., 2002; Worm et al., 2002a; Meng et al., 2003).

After the first description of HEV in swine (Balayan et al., 1990; Clayson et al., 1995a; Meng et al., 1997), an initial epidemiological survey in North America reported a higher prevalence of anti-HEV antibodies among swine veterinarians (26%) than among blood donors (18%) (Meng et al., 2002). A further study described a 35, 11 and 2.5% seroprevalence among swine, swine workers and non-swine workers, respectively (Whiters et al., 2002). Recently, a Chinese study has reported that swine workers have a 74% higher risk of HEV infection than people engaged in other occupations (Zheng et al., 2006). In contrast, no statistical difference in anti-HEV antibodies prevalence has been noted between pig farmers (13.0%) and control subjects (9.3%) in Sweden (Olsen et al., 2006). In any case, it should be noted that differences in study design (population features, health status, demographical variables, etc…) make difficult the comparison of the reported data, and that there are contradictory results about the reliability of the anti-HEV detection test used in the different studies (Worm et al., 2002a; Emerson and Purcell, 2003; Schlauder, 2004). Hence, analyses of well-selected population with standardized reagents are needed to have a more clear understanding of the actual incidence of HEV infection.

## *General features of the hepatitis E virus (HEV) with a special emphasis to the genomic organization*

The identity of the causative agent of hepatitis E was first described in 1990 (Reyes et al., 1990). One year latter, the entire sequence of the viral genome was published (Tam et al., 1991). HEV was provisionally classified as a member of the *Caliciviridae* family, but it is now ascribed to a separate family, *Hepeviridae*, in the

prototypic genus *Hepevirus* (Mayo and Ball, 2006). HEV is a spherical, non-enveloped viral particle of around 32–34 nm in diameter. The genome is a ssRNA molecule of positive polarity of approximately 7.2 Kb containing 3 overlapping ORF and a 3′poly (A) tail (Worm et al., 2002b; Emerson and Purcell, 2003; Schlauder, 2004). AU :12 AU :13

*In vitro* analysis suggested that HEV RNA is capped at the 5′end (Kabrane-Lazizi et al., 1999a). After a non-coding region of 27–35 nucleotides (nt), ORF-1 encodes about 1693 aa encompassing non-structural proteins with enzymatic activity that are involved in viral replication, transcription and protein processing, including the viral replicase (Emerson and Purcell, 2003). ORF-2 extends 1980 nt, terminating 65 nt upstream of the poly-A tail, and renders a 660 aa protein likely representing the structural capsid protein(s) (Tam et al., 1991). *In vitro* experiments suggested that ORF-2 protein is synthesized as a large glycoprotein precursor of around 88 kDa, which is cleaved into the mature protein (Jameel et al., 1996, Zafrullah et al., 1999). ORF-2 protein contains epitopes that induce neutralizing antibodies and are mainly located near the carboxi-end (Tam et al., 1991). ORF-3 overlaps the 5′end of ORF-1 by only 1 nt and ORF-2 by 328 nt. It encodes a 123 aa protein which is post-translationally modified by phosphorylation giving a mature protein of around 13.5 kDa of unknown function (Emerson and Purcell, 2003). This phosphoprotein is associated with the hepatocellular cytoskeleton (Zafrullah et al., 1997) and form a complex with capsid protein of ORF-2 and, thus, it is believed to be involved in the assembly of the viral particle (Jameel et al., 1996). However, it has been recently shown that, in contrast to its requirement *in vivo*, ORF-3 protein is not required for infection of Huh-7 cells or production of infectious virus *in vitro* (Emerson et al., 2006). ORF-3 may also have regulatory functions implicated in modulation of cell signaling (Emerson and Purcell, 2003). In addition, ORF-3 protein also beards neutralizing epitopes near its 3′end (Tam et al., 1991). In any case, it should be noted that the lack of a suitable and efficient cell culture system for replication of HEV has hampered the study of the viral life cycle (Emerson and Purcell, 2003).

### *Genetic variation*

The genome sequence of HEV is quite stable (Arankalle et al., 1999). A high genomic homology is found among isolates from the same outbreak, and serial passages in animal models did not result in genetic drift (Worm et al., 2002b; Schlauder, 2004). However, data supporting a quasispecies organization of HEV genome during epidemics have also been reported (Grandadam et al., 2004). Additionally, isolates from different geographical regions are relatively diverse. Based on this genomic heterogeneity, HEV has been classified into four different genotypes (Worm et al., 2002b; Meng, 2003; Schlauder, 2004). Genotype I is mainly presented in endemic areas from Asia and Africa. Genotype II includes the Mexican isolates and some Nigerian strains. Isolates from regions considered as non-endemics (USA, Spain, Italy, Greece, etc.) represent a more diverse cluster of

sequences and are grouped into genotype III. Finally, genotype IV includes isolates from China. Besides this genotypic diversity, no evidence of serological heterogeneity has been reported and, therefore, it seems that there is only one HEV serotype.

## *HEV transmision*

### *Waterborne transmission*

Epidemics of hepatitis E in endemic areas are usually due to fecally contaminated water (Aggarwal and Naik, 1994) and most outbreaks can be traced back to contaminated water sources (Smith, 2001). Adequate circumstances for HEV epidemics arise when raw sewage enters in contact with water reservoirs during heavy rain sessions, floods, monsoons, etc. For instance, heavy and flooding rains preceded the Indian epidemic of the 1950s (Vishwanathan, 1957; Khuroo and Kamili, 1994). AU :14 In most instances, people affected by HEV outbreaks lives near rivers with inadequate sanitary conditions (Bile et al., 1994) and a high incidence of HEV seropositivity has been correlated with the use of non-boiled river water for drinking, cooking and washing. Likewise, refugees and people living in urban crowded slums and camps are at increased risk for fecal-oral transmitted diseases, including HEV infection (Khuroo and Kamili, 1994; Mast et al., 1994), as it has been recently demonstrated in the displaced population from Darfur (Sudan) where, in 6 months, 2.621 hepatitis E cases were recorded (attack rate 3.3%). The case-fatality rate was 1.7%, with 45 deaths, including 19 pregnant women (Guthmann et al., 2006).

HEV was detected in all sewage influent samples and in 67–89% of effluent samples from sewage treatment plants in Madras, India, showing that treatment was not as effective as it should be. Viral particles have also been detected in sewage from industrialized countries (Pina et al., 2000). HEV RNA was detected in a pretreated sewage sample collected in Washington, DC, and it showed a very high homology with human and swine isolates from the US (Clemente-Casares et al., 2003). In contrast, no HEV RNA has been detected in drinking and surface waters collected from pig farms where HEV was present (Kasorndorkbua et al., 2005).

### *Person-to-person transmission*

Person-to-person transmission seems to be low (Aggarwal and Naik, 1994; Bile et al., 1994; Mast et al., 1994). Secondary cases among household members of patients with documented HEV infection occurs in 1–2% (Aggarwal and Naik, 1994). Person-to-person transmission in hospital settings has been described, although results about the incidence of HEV infection in hospitalized patients and in people that received contaminated blood are inconclusive (Smith et al., 2001). Data on mother-to-child transmission rates of HEV are quite variable, ranging between 30 and 100% (Khuroo et al., 1995; Kumar et al., 2004). Additionally, it has been reported that up to 2/3rd of pregnant HEV-infected women may have preterm delivery

(Kumar et al., 2004). HEV RNA has also been detected in the blood of newborns at a time when no virus was detectable in the mother (Khuroo et al., 1995).

*Foodborne transmission*

Washing, irrigating and processing of food with HEV-contaminated water could lead to HEV outbreaks if the food is eaten uncooked. Food manipulation by an HEV-infected person may also transmit the disease. Acute hepatitis E in Sicily (Italy) was attributed to contaminated shellfish consumption (Cacopardo et al., 1997), and a case of hepatitis E after ingestion of Chinese medicinal herbs has also been reported (Ishikawa et al., 1995). Likewise, sporadic acute or fulminant hepatitis has been linked to uncooked pig liver and wild boar meat consumption in Japan (Yazaki et al., 2003; Li et al., 2005). Finally, a clear demonstration of acute HEV infection after consumption of HEV-infected uncooked deer meat has been reported (Tei et al., 2003). In this latter study, 4 out of 5 individuals who ate the infected meat presented hepatitis, while the 3 other members of the families who did not eat it were not infected. One child who was not infected claimed to have eaten a very small portion of deer meat, suggesting that HEV infection is dose-dependant. AU :15 Sequence analysis of HEV RNA from patients and from frozen leftover deer meat showed a 100% similarity (Tei et al., 2003).

*Zoonotical transmission*

Presence of anti-HEV antibodies in pigs and characterization of swine HEV were first described in the 1990s (Balayan et al., 1990; Clayson et al., 1995a; Meng et al., 1997). Later on, experimental infection of pigs with either swine HEV or human isolates was achieved, and showed that infected animals presented viremia and shed virus in feces, although no clinical or biochemical signs of disease were observed (Balayan et al., 1990; Meng et al., 1998; Halbur et al., 2001). After that, several evidences have raised the hypothesis of a zoonotic potential for HEV and its possible risk in xenotransplantation (Meng, 2003). For instance, HEV has been detected in sewage polluted with pig feces (Pina et al., 2000), people drinking water from downstream of pig farms seem to have a higher risk of HEV infection (Zheng et al., 2006) as do workers engaged in occupations related to swine farming (Meng, 2003; Zheng et al., 2006), and anti-HEV antibodies have been found in swine herds from endemic and non-endemic areas (Emerson and Purcell, 2003; Meng, 2003; Goens and Purdue, 2004). Furthermore, in general, swine isolates are genetically more closely related to human HEV strains of the same geographical region than to swine strains of other parts of the world (Meng et al., 1997; Meng, 2002; Meng et al., 2003). Finally, as aforementioned, HEV infection in humans after ingestion of HEV-infected raw deer meat has been demonstrated (Tei et al., 2003).

Besides pigs, specific anti-HEV antibodies and HEV strains have also been detected in rodents (Clayson et al., 1995a; Kabrane-Lazizi et al., 1999b), wild boar (Matsuda et al., 2003), donkeys (Guthmann et al., 2006), chickens, cattle and dogs

(Meng, 2003; Goens and Purdue, 2004). More recently, an avian HEV has been described (Haqshenas et al., 2001) and, although it is genetically less related to human HEV than swine isolates, it shares antigenic epitopes with both of them (Haqshenas et al., 2002). All these observations have strengthened the zoonotic potential of HEV, but the assessment of a zoonotical transmission to humans through animal waste still needs further evaluation.

## *Diagnosis*

### *Serological diagnosis*

Enzyme-linked immunosorbent assay (EIA) is the main diagnostic tool to detect anti-HEV IgG and IgM (Worm et al., 2002b). In general, a positive result for anti-HEV IgM indicates acute disease, however, to avoid false negative results, testing should be done in the acute phase of the infection. Although detection of anti-HEV IgG is not conclusive of HEV infection, a high IgG titer or increasing titers in consecutive samples, support the diagnosis of acute hepatitis E.

Several antigenic domains have been identified in the three ORFs of HEV. Based on this information, different synthetic peptides and recombinant proteins derived from the carboxi-end of ORF-2 and/or ORF-3 have been assayed for specific antibody detection (Worm et al., 2002a). Studies carried out with these tests have reported a relatively high seroprevalence in non-endemic countries, raising concerns about the possible detection of non-specific cross-reactive antibodies (Mast et al., 1998; Worm et al., 2002a). However, a blind comparison of a test based on a recombinant ORF-2 protein showed that it was 98% specific for anti-HEV (Mast et al., 1998). When this approach was applied to sera from different regions of the world, it confirmed the previous results obtained with other assays (Emerson and Purcell, 2003). At present, the few commercially available EIAs kits are based on the Mexican and/or the Burmese prototypes, and although a recent comparison of different tests using outbreak samples has shown that they can be highly specific and sensitive (Myint et al., 2006), their reliability still needs to be fully confirmed worldwide.

### *Molecular detection*

Detection of HEV by RT-PCR is indicative of active infection. The availability of an increased number of HEV sequences from different sources and geographical regions has enabled the design of specific oligonucleotide primers that match conserved regions of the HEV genome and allows the detection of HEV in acute phase sera, stools and contaminated water and sewage (Schlauder et al., 2004). Several conventional "in-house" RT-PCR assays have been published for detection of HEV in serum, feces and bile of infected individuals (Smith, 2001). For water analysis, efficient concentration procedures and highly sensitive detection methods are required for viral detection (Jiménez-Clavero et al., 2005b; and chapter by

Wyn-Jones). Recently, several real-time RT-PCR detection methods have been described (Mansuy et al., 2004; Orrù et al., 2004; Enouf et al., 2006; Jothikumar et al., 2006). Using an internal control and spiked water samples, detection of as few as 4 genome equivalent copies of HEV plasmid DNA and of 0.12 pig $ID_{50}$ of swine HEV has been achieved (Jothikumar et al., 2006). Development of a quantitative, broadly reactive, quick, easy and reproducible HEV detection method would be of special interest for testing water and environmental samples, and may allow tracking of the polluting sources. Recently, as few as 100 fM of an ORF-2 amplicon were detected using an HEV specific microarray (Liu et al., 2006). Ideally, development of a microarray assay able to detect as much waterborne pathogens as possible in a single reaction would greatly improved our current capacity for detection of water pollutants representing human and/or animal health risk.

AU :16

### *Prevention of hepatitis E*

The feasibility of HEV vaccines is based on several evidences: (i) specific antibodies are raised after HEV infection; (ii) HEV infected people are usually protected following epidemics; and (iii) animal experimentation has shown that passive immune prophylaxis induces humoral immunity (Emerson and Purcell, 2001; Wang and Zhuang, 2004). Additionally, only one HEV serotype has been described, thus, production of a broadly cross-reactive vaccine should be possible. Such a vaccine would be useful in protection against HEV infection, mainly in pregnant women and in people from endemic regions and travelers to these areas. Nevertheless, the lack of a susceptible cell culture system has hampered the development of live attenuated or killed vaccines (Wang and Zhuang, 2004) and hence no commercial vaccines against HEV are available.

To date, most research on HEV vaccines is focused on ORF-2-derived proteins or peptides that contain neutralizing epitopes common to different genotypes (Emerson and Purcell, 2001; Meng, 2001; Worm et al., 2002a; Wang and Zhuang, 2004). Several ORF-2 vaccine candidate products have been expressed in insect, prokaryotic, yeast, animal and plant cells (Emerson and Purcell, 2001; Wang and Zhuang, 2004). Animal experimentation has shown that administration of some of these ORF-2 recombinant proteins protected against homologous and heterologous challenge (Purdy et al., 1993; Tsarev et al., 1994; Tsarev et al., 1997; Im et al., 2001). DNA immunization of mice with an HEV-cDNA elicits high titers of specific anti-HEV antibodies (He et al., 1997), immunologic memory (He et al., 2001) and protection in cynomolgus macaques (Kamili et al., 2004). Truncated ORF-2 protein expressed in baculovirus spontaneously assembles into viral-like particles (VLPs) and are also good immunogens (Li et al., 2001). A recombinant HEV baculovirus vaccine candidate that protects against intravenous administration of heterologous HEV strains has entered into preclinical trials (Stevenson, 2000; Zhang et al., 2002; Purcell et al., 2003).

## *Concluding remarks*

Enterically transmitted hepatitis represent by large the most common manifestation of acute hepatitis worldwide. Regarding hepatitis A, although the increase in living standards and public health sanitation are greatly contributing to a decrease in its global incidence, the total number of cases per year is still extremely high with estimations up to the scale of millions. Overall the situation encourages policies of immunization, but in spite of the availability of vaccines, their use in highly endemic regions may be limited on the basis of their cost and even on the basis of medical and molecular aspects. Thus in these areas prevention should, at least, rely on the implementation of effective control measures such as water sanitation and virus monitoring.

In the case of hepatitis E, the disease that was restricted to endemic areas is now been increasingly reported also in regions considered as non-endemic. However, it remains unclear whether this increase is related to the emergence of the pathogen or to the new epidemiological and public health interest in this virus and the availability of diagnostic procedures. This concern derives from the associated mortality in pregnant women, as well as the morbidity and disability in the general population, mainly in endemic areas. Since HAV and HEV are transmitted by contaminated waters, improvement of water quality by proper sewage disposal and water treatment is very important in preventing spread of these infections.

Although it may appear unlikely that infectious hepatitis outbreaks occur in industrialized countries with properly treated waters and sewages and good sanitation and hygienic conditions, potential risk derived form consumption of vegetables, fruits and other products imported from endemic regions exist. Bivalve mollusks grown and harvested in polluted waters represent a particular potential threat due to their capacity to filter large volumes of water and accumulate the viruses in their edible tissues.

The possible zoonotic transmission of HEV may also contribute to the continuous spread of the virus, despite improved sanitation, and calls for a more deep knowledge of HEV prevalence in animals in order to prevent hepatitis E transmission.

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