Hepatitis E in the Immune-Compromised Patient

Hepatitis E in de immuun-gecompromitteerde patiënt

Suzan D. Pas, 2015

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IF IT IS TO BE, IT IS UP TO ME
William H. Johnson

Abbreviations

γ-GT	gamma glutamyl transpeptidase
A549	adenocarcinoma human alveolar basal epithelial cells
aa	amino acid
AASLD	American association for study of liver diseases
ALL	acute lymphoblastic leukemia
alloHSCT	allogeneic haemopoietic stem cell transplantation
ALT / ALAT	alanine amino transferase
AML	acute myeloid leukemia
AST / ASAT	aspartaat amino transferase
AUC	area under the curve
BSA	bovine albumin fraction V
CI	confidence interval
CLL	chronic lymphocytic leukemia
CMV	cytomegalovirus
CPE	cytopathogenic effect
Ct(-value)	cycle threshold (value)
DAB	3,3'-diaminobenzidine
DAPI	4,6-diamidino-2-phenylindole
DILI	drug induced liver injury
DMEM	Dulbecco's modified eagles medium
EASL	European association for study of the liver
EBV	Epstein-Barr virus
EGFR	epidermal growth factor receptor
EDTA-plasma	Ethylenediaminetetraacetic acid - plasma
EIA	immunosorbent assay
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
FDA	Food and drug administration
GBS	Guillain-Barré syndrome
Gt	genotype
GVHD	graft versus host disease
HAI	histology activity index
HAV	hepatitis A virus
HBsAg+	hepatitis B surface antigen positive
HBV	hepatitis B virus
HCV	hepatitis C virus

HEV	hepatitis E virus
HSPG	heparin sulfate proteoglycans
HIV	human immunodeficiency virus
HTX	heart transplantation
i.v.	intravenous
IgG	immunoglobulin G
IgM	immunoglobulin M
IU	international units
JAK/STAT	Janus kinase / signal transducer and activator of transcription
Kb	kilobases
LIMS	laboratory information management system
LLOD	Lower limit of detection
LTX	liver transplantation
LuTX	lung transplantation
MAPK / ERK	mitogen activating phosphokinase / extracellular signal-regulated kinases
MDS	myelodysplastic syndrome
MM	multiple myeloma
METC	medisch ethische commissie
MUD	matched unrelated donor
NAT	nucleic acid testing
NHL	non-Hodgkin's lymphoma
Nm	nanometer
KTX	kidney transplantation
OD	optical density
ORF	open reading frame
Peg-INF	pegylated interferon
PCP	papain-like cysteine protease
p.i.	post infection
PBS	phosphate buffered saline
pORF	protein of open reading frame
PSAP motif	proline-serine-alanine-proline motif
R-CHOP	rituximab - cyclophosphamide, hydroxydaunorubicin, oncovin, prednisolone
PRP	polyprotein domain
RdRp	RNA dependent RNA polymerase
RNA	ribo nucleic acid
ROC	receiver operator curve

RT-PCR	reverse transcription - polymerase chain reaction
S/N	signal to noise
SD	standard deviation
SIB	sibling
SOT	solid organ transplant
SF1	superfamily 1
TCID50	50% tissue culture infectious dose
UBC	umbilical cord blood
ULN	upper limit of normal
UK	United Kingdom
uPA/NOG	urokinase-type plasminogen activator / NOD/Shi-scid/IL-2Rγnull
USA	United States of America
UTR	untranslated regions
WHO	world health organization

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Chapter 1

General introduction

Taken in part from

Hepatitis E virus: current concepts and future perspectives

Harry R. Dalton, **Suzan D. Pas**, Richie G. Madden
and Annemiek A. van der Eijk

Current Infectious Disease Reports (2014) 16:399.

HISTORY OF HEPATITIS E VIRUS DISCOVERY

Hepatitis E virus (HEV) has probably been around for quite some time. Already in the 18th century outbreaks of jaundice, with coinciding fatalities in pregnant women (a hallmark of genotype (gt) 1-2 hepatitis E virus infections) were reported in Europe[1]. Following an epidemic of non-A non-B hepatitis in the late 70s[2], hepatitis E virus (HEV) was discovered as a virus transmitted via the feco-oral route, through experimental infection of a volunteer in 1983[3] with a pool of stools from patients suffering from hepatitis. The virus was visualized by electron microscopy and subsequently cynomolgus macaques (*Macaca fascicularis)* were infected. The HEV genome was sequenced in 1990[4, 5], after which its protein functions were characterized[6]. In 2009, the structure of HEV was further elucidated through X-ray crystallography.[7]

It has been estimated that HEV gt 1 and gt 2 infection alone causes >3 million symptomatic cases of acute hepatitis E each year that result in approximately 70,000 deaths[8]. For many years, hepatitis E was considered a disease of certain developing countries, that was associated with mortality in pregnant women. Our understanding of HEV infection has changed radically in the past decade, with HEV being currently considered a global threat to human health.

TAXONOMY

Only 12 years after its discovery in 1983 HEV was assigned as a family member of the family *Caliciviridae,* genus *Calicivirus* on the basis of clinical symptoms and structural features. It soon became clear, however, that the virus did not have all intrinsic features of a calicivirus and as a result its taxonomical position was changed in 1998, grouping this species into an unassigned family and within the genus *hepatitis E-like viruses*. In 2004, the genus *hepatitis E-like viruses* was renamed to *Hepevirus*, which was assigned to the family *Hepeviridae* in 2008[9]. Recently, taxonomic division of the family *Hepeviridae* was completely changed as a result of the discovery of multiple HEVs in different hosts and the contradictory taxonomical positioning proposed by researchers. The family *Hepeviridae* now comprises the genera, *Orthohepevirus* and *Piscihepevirus* and an additional, unconfirmed genus *Hepelivirus,* of which the genus taxonomic positioning is based on partial RdRp sequences only[10] (Figure 1).

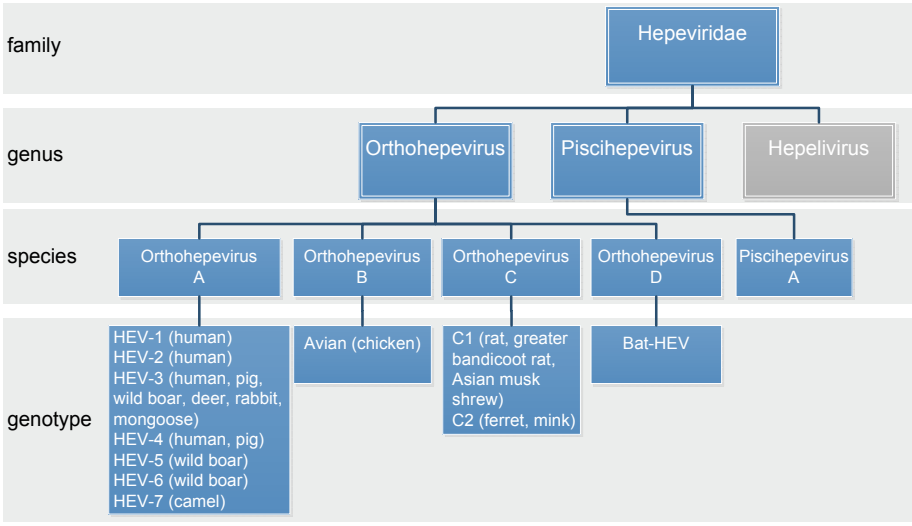

Figure 1 Taxonomy of HEV. Blue boxes indicate accepted taxonomic positioning, grey box is currently unconfirmed. The species *Piscihepevirus A* was previously called cut throat trout virus. Although ICTV does not specify classification on the level of genotype, it is illustrated in this figure for historical reference.

The recent reclassification of HEV genotypes was defined on basis of ORF1 motifs (methyltransferase, helicase and RdRP) and concatenated ORF1/ORF2 sequences. Within the species *Orthohepevirus A*, there are four genotypes, HEV gt 1-4, known to infect humans, which can be divided into two groups (genotype 1-2 and 3-4) on basis of host, epidemiology and geographical distribution (Figure 1 and Figure 2). Among these four genotypes the nucleic acid identity ranges between 73-77%, with >83% within a genotype[11]. The utility of HEV subtypes within HEV gt 3 is a subject of debate. Inconsistencies of the previously suggested 24 subtypes[12] have been observed [13, 14], probably due to the small part of ORF2 not being representative for the complete genome sequence variation. Therefore the ICTV recommends not to use sub-genotyping, but rather assignments as 'clades'. HEV gt 1-4 belong to one serotype[15].

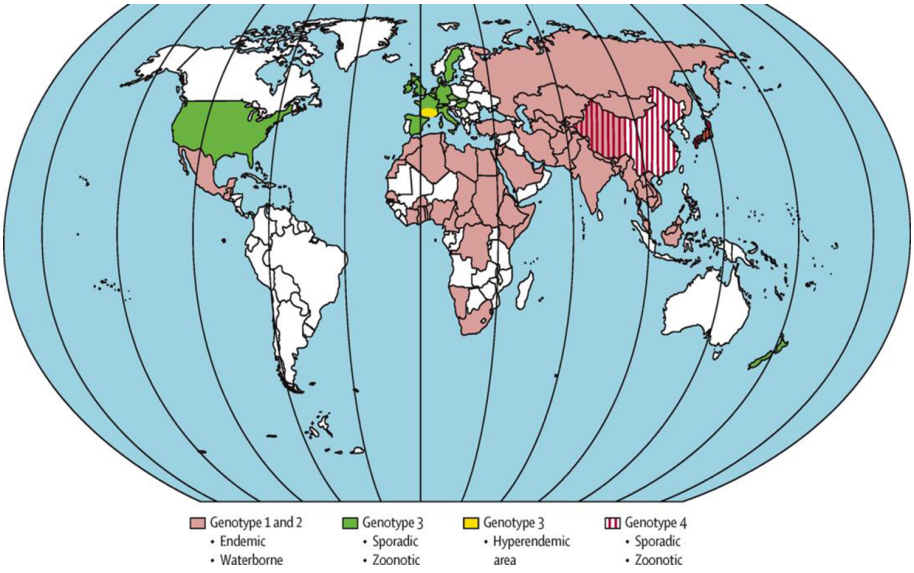

Figure 2 Predominant geographical distribution of HEV[16]

HEV REPLICATION STRATEGY

The hepatitis E virion is non-enveloped and 27-34 nm in size. The viral genome is single stranded positive sense RNA of ~7.2Kb, grouped into group IV positive single stranded RNA viruses. The genome consists of a 5′ m^7G-cap, short 5′ untranslated regions (UTR), 3 partially overlapping open reading frames (ORF) of positive polarity, a short 3′UTR and a 3′ polyA tail [5]. The life cycle of HEV is not completely understood, but studies performed in recent years have provided important insights into its replication strategy.

The virus replication cycle (Figure 3) starts with virus capture on the host cell by non-specific sticky molecules called heparin sulfate proteoglycans (HSPG)[17] acting as attachment factors (Figure 3, step 1). The virus is thought to enter the host cell by a so far unknown receptor[18] (Figure 3, step 2,3), after which the positive stranded genomic RNA is released (Figure 3, step 4) into the cytosol by an unknown process after which ORF1 is immediately transcribed.

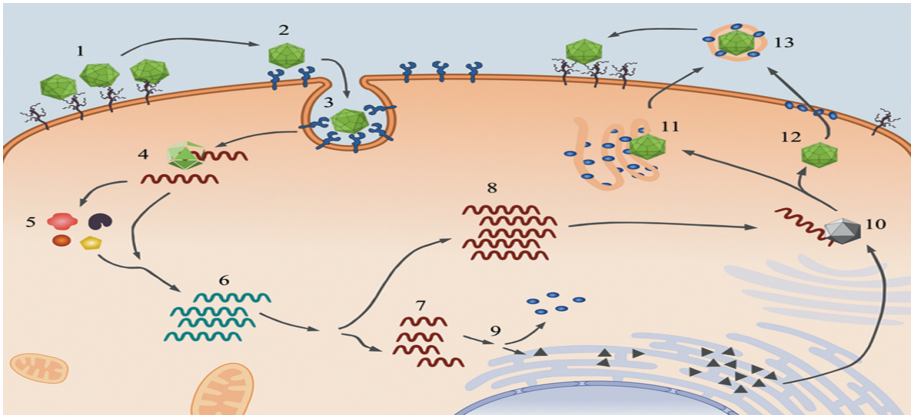

Figure 3 HEV replication cycle[18] **© Georg Thieme Verlag KG**

ORF1 encodes a nonstructural polyprotein of 1693 aminoacids, with methyltransferase, papain-like cysteine protease (PCP), RNA helicase and RNA-dependent RNA polymerase (RdRP) activity[4, 6] (Figure 3, step 5 and Figure 4). PCPs are found in a wide range of positive stranded RNA viruses and mediate the processing of non-functional proteins, whereby the X-or macro domain (Figure 4) flanks the PCP coding sequence[19]. HEV RNA helicase belongs to the superfamily class 1 (SF1) of helicases, which mediate the unwinding of 5'-overhangs (5'-3'), have RNA triphosphatase (RTPase) activity and thereby play an important role in forming viral cap[20]. The HEV RdRP belongs to the supergroup III of viral RNA polymerases and is involved in the first steps of HEV genome replication, transcribing the positive sense genome into negative sense RNA (Figure 3, step 6), which is subsequently used as template for generation of positive sense genomic and 2.2 Kb subgenomic RNA (Figure 3, step 8 and Figure 4).

Besides the known and functionally established protein, ORF1 also encodes a Y domain, polyproline (PRP or V (variable)) domain and X (or macro) domain (with a highly conserved glycine triade, pink triangle Figure 4). The function of the Y domain is unknown, however the polyproline region (V, Figure 4) seems to play a role in viral adaptation[21] and is also designated as a protein hinge region[6].

Once +ssRNA is transcribed into –ssRNA (Figure 3, step 7), a precursor ORF2 protein is translocated to the endoplasmic reticulum and undergoes glycosylation at N137, N310 and N547 aminoacid residues (Figure 3, step 9 and Figure 4, blue stars)[22]. This glycosylation has been shown to be crucial in the formation of infectious HEV particles[23]. ORF2 encodes the viral capsid protein of 660

aminoacids / 88kDa, containing three distinct domains, namely shell (S, aa129-319), middle (M, aa230-455) and protruding (P, aa456-606) domains[24]. pORF2 is a multifunctional protein, as it is involved in virion assembly[25] (encapsidating the viral genome (Figure 3, step 10)), host-virus interaction by heparin sulfate proteoglycans (syndecans)[17] during the viral capture, and harbors virus neutralizing epitopes within the aa458-607 region of the P domain[24, 26-28]. The pE2 domain (aa394-606) forms tight homodimers, of which aa597-602 of the dimeric domain (aa459-606) is involved in dimerization and both aa394-459 and aa607-660 have a stabilizing function[25]. This homodimerization is essential for virus-host interaction. A truncated ORF2 peptide p239 (aa368-606) forms 23 nm virus like particles and is used as antigen in the currently available hepatitis E virus vaccine.

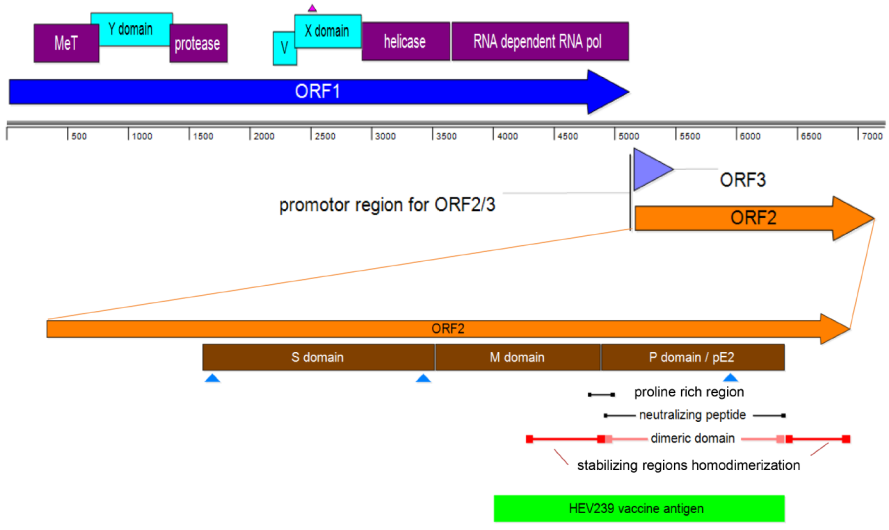

Figure 4 HEV genomic organization[6, 18, 29].

ORF3 was previously thought to be transcribed into a 123aa protein[30]. However, Graff *et al*[31] described a bicistronic 2.2Kb subgenomic RNA fragment to encode both pORF3 and pORF2 in separate reading frames. It was shown that not the first, but the third in-frame AUG initiated translation of ORF3. Therefore, pORF3 was found to be 9 aminoacids smaller (114aa) than previously described. The first four amino acids of ORF3 (MNNM) are thought to be the promoter[31], as they are highly conserved within the species and shown to be a cis-reactive element[32]. pORF3, a 13 kDa phosphoprotein, contains two hydrophobic domains D1 (aa7-

23), D2 (aa28-53) and two proline rich domains, P1 (aa66-77) and P2 (aa95-111)[30, 31]. These proline rich motifs (PXXP) interact with Scr homology (SH3) domains of host intracellular signaling proteins and bind and inhibit mitogen activating phosphokinase (MAPK). These observations, and the association of pORF3 to epidermal growth factor receptor (EGFR)[33], suggest that pORF3 regulates transcription by interacting in MAPK/ERK and JAK/STAT intracellular signaling pathway thereby hampering immune activation[34]. In addition, studies have shown that the pORF3 aa1-32 interact with the cytoskeleton (tubulin)[35], a part of pORF3 is able to interact with non-glycosylated pORF2 (by aa58-81)[36] and that a PSAP motif (aa95-98) within the P2 domain associates with lipids of the vacuolar protein sorting pathway[37]. These findings suggest pORF3 plays an import role in the replication cycle through virion release by budding (Figure 3, step 11,12). However, it is still unclear how the lipid-ORF3 complex is removed from the virion before infecting new host cells (Figure 3, step 13).

DIAGNOSING HEV INFECTION

Confirmation of HEV infection relies on the detection of HEV specific serum IgM and IgG antibodies and/or HEV RNA testing in serum or feces[38]. Until very recently HEV has proven difficult to culture and this method is not used for diagnostic purposes; moreover real-time PCR techniques are more sensitive than virus culture. In immune-compromised patients antibody production may be delayed or absent[39], however this has not been studied in detail for HEV infections. In immune-competent individuals, there is a narrow window (mean 28, range 17-48 days)[40] in which HEV RNA can be detected in serum and/or feces. Therefore, a combination of serology and RNA detection is required to diagnose HEV infection in such individuals. The diagnosis of reinfection can be more problematic, and may be more common than currently appreciated. Such patients are typically anti-HEV IgM negative, and so the diagnosis depends on the demonstration of HEV RNA by PCR[16]. When patients with reinfection present after the viremic period they will be PCR and anti-HEV IgM negative, but anti-HEV IgG positive. These results are indistinguishable from those found in more distant previous infection, and so the only way of making a diagnosis of reinfection in this situation is by demonstrating IgG antibodies of high avidity and/or a rising titer of IgG in a convalescent sample. However these additional serological assays are rarely performed in routine clinical practice.

HEV serology

Performance of commercial and in house anti-HEV IgM and IgG assays have been compared and there appears to be discordance between the results of some assays. Some anti-HEV IgG commercial assays have low sensitivities, particularly to detect antibodies raised to HEV infection that has occurred more than 1 year before[41]. This almost certainly has resulted in an underestimation of HEV prevalence in many of the early studies. Differences in assay design are considerable and include: (1) the format used, like direct, indirect, or a μ chain based capture assay, (2) the genotype of the antigens used. In most ELISA's only gt 1 and 2 recombinant antigens are used, while only one assay currently uses gt 1 and 3 antigens in their assays and, (3) the corresponding structural regions of the recombinant antigens which currently are all ORF2 $\pm$ ORF3 and, (4) the presently preferred use of antigen dimers.

There is limited validation data on currently used serological assays. One IgG assay has been partially validated against convalescent sera taken up to 7 years following PCR-proven acute infection, and was shown to have a sensitivity of 98%, although it's specificity has not been completely confirmed[41]. Further validation of existing serological assays for detecting HEV infection is urgently needed.

HEV molecular testing

Until recently, molecular testing for HEV has been problematic, as only a range of in-house assays have been used. A comparison among 20 European laboratories showed a wide variability in the results of quantitative assays[42], highlighting that international standardization is urgently needed. In addition HEV genotyping is important for research purposes and epidemiologic studies[43], Conventional RT-PCR products are used for genotyping HEV on different genome segments. Partial ORF2 sequences have historically been widely used[44], while Zhai *et al*[45] showed that a 306bp region of RdRp (ORF1) was statically most representative for the complete HEV genome flanked by conserved primer sites.

EPIDEMIOLOGY

The epidemiology of HEV gt 1-2 infections is distinct from HEV gt 3-4 infections. In resource-low countries, with poor hygiene, hepatitis E virus gt 1 causes a self-limiting hepatitis in young adults, with a case fatality rate of 0.2-4%. However mortality of gt 1 infections in pregnant females ranges from 25 to 31%[16, 46, 47]. HEV gt 3 is found in pigs worldwide, and gt 4 is found in pigs in China and Japan. Although HEV infections in swine herds are asymptomatic, infected pigs excrete large quantities of HEV in their feces. The pig is considered to be a primary host and there is a close similarity between HEV strains obtained from pigs and humans[48]. HEV is highly infectious in pig herds with a basic reproductive ratio (R_0) ranging up to 8. Once HEV has been introduced into a pig herd, infection will soon be wide-spread[49, 50]. HEV RNA was detected in 33-55% of fecal samples of Dutch pig farms, and in 6.5% of the commercially available porcine livers[51, 52].
HEV has also been found in many other animals including deer and rabbits[53], which can also be a source of human infection. In comparison to pigs, they are a less important reservoir for human infection. HEV has also been documented in a range of other mammals, including rats, bats, and ferrets[54-56]. It is uncertain if these animal reservoirs pose a risk to human health. For example rat HEV is not transmissible to rhesus monkeys, suggesting that it is unlikely to be pathogenic to primates, including humans [57].
There are a number of possible routes of infection (Figure 5), but in most human hepatitis E cases, it is impossible to determine the source or route of infection. This is largely due to the incubation period of approximately 6 weeks, which period is often too long to remember what was consumed and to identify HEV RNA in consumed products. However, consumption of infected pork products has been well documented. HEV has been found in retail pork in grocery stores in several European countries, Japan and the United States of America (USA). In 2012, HEV gt 3 was identified throughout the human food chain in the United Kingdom (UK) and shown to be present in 10% of tested retail sausages[58]. HEV requires cooking temperatures of 71°C for 20 minutes to be inactivated, which is much longer than sausages usually are cooked[59]. HEV has also been found in several food products like traditional French air-dried pig liver sausages, which are not cooked at all[60]. A recent case control study from the UK suggested that processed pork products such as ham and pork pies can also be a source of infection[61].

Another source of infection is infected water (Figure 5) as HEV has been found in surface fresh and sea water[62]. In southwest England, 50% of cases of hepatitis E were found to live within 2 km from the coast[63], which led to the speculation that recreational use of HEV contaminated water is a source of HEV infection. In addition, a recent study found HEV in shellfish[64].

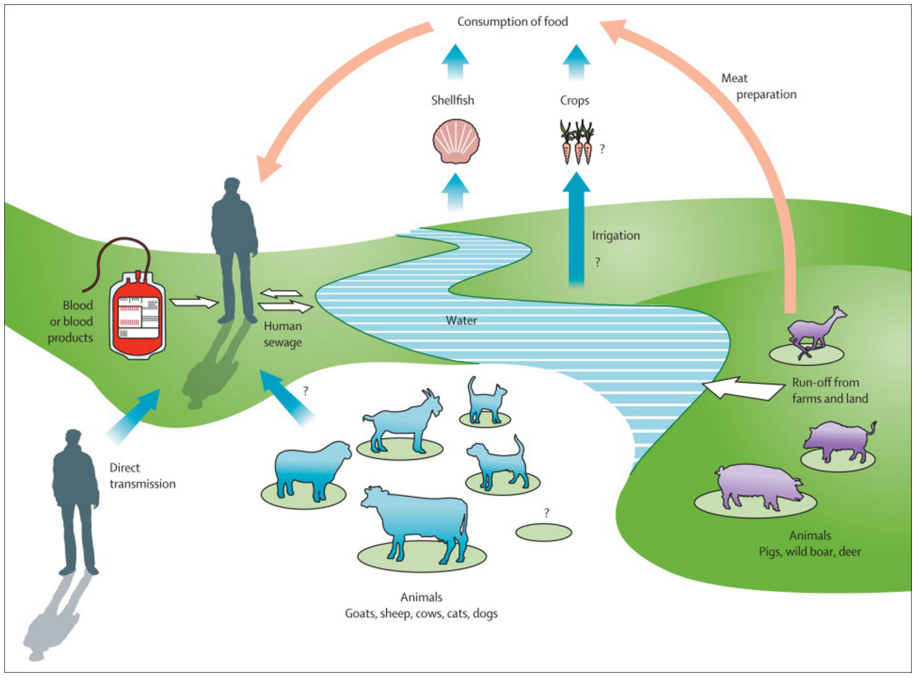

Figure 5 HEV transmission routes[16]

It has previously been documented that the sero-prevalence of anti-HEV IgG in many developed countries was low, ranging from 1 to 2%. However these studies may be flawed, as assays of poor sensitivity were used (see above). Also, the incidence of hepatitis E among humans has been poorly documented, but undoubtedly varies between and within countries and over time. The incidence in the UK has been estimated to be about 0.2%[65], and in the USA about 0.7%[66]. With a population of about 316 million, this implies that over 2.2 million infections occur in the USA annually. Virtually all of these are asymptomatic or unrecognized, as no FDA approved HEV diagnostics are currently available in the USA.

CLINICAL ASPECTS OF HEPATITIS E

Acute HEV gt 3 and HEV gt 4 infections in immune-competent persons are usually self-limiting lasting 4–6 weeks, requiring no treatment. Asymptomatic infection is very common, in an outbreak of hepatitis E gt 3 on a cruise ship, 67% of the infected individuals had no symptoms[67]. However in sporadic cases acute HEV infection may develop into fulminant hepatitis, that may even require liver transplantation[95]. Symptoms of hepatitis E are largely non-specific and indistinguishable from other causes of viral hepatitis, like jaundice, anorexia, lethargy, abdominal pain, vomiting, fever, fatigue. Less common symptoms are myalgia, pruritis, weight loss, headaches, arthralgia and neurological symptoms[68].

Notably, in immune-compromised patients like solid organ transplant patients HEV gt 3 infection may become chronic. Up to this date no cases of chronic hepatitis E have been reported for genotypes 1, 2 and 4 infections[16]. A chronic HEV infection is defined by HEV viremia in plasma or stool detected by RT-PCR for 6 months or more. Persistent chronic infection has been reported in immune-compromised patients, with one study reporting 57% of liver transplant patients developing chronic hepatitis[69]. Treatment with tacrolimus, an immunosuppressive drugs inhibiting the T-cell signal transduction and interleukin-2 production[70], has been found to be the risk factor for development of chronic hepatitis E virus infections[71]. Still little is known about the incidence and clinical consequences of chronic HEV infection and its clinical consequences in immune-compromised patients in The Netherlands.

TREATMENT OF HEPATITIS E

Currently there are no WHO nor EASL / AASLD guidelines for treatment of hepatitis E. Most cases of acute hepatitis E infection are self-limiting, requiring only symptomatic or no treatment. However a minority of the patients develop fulminant hepatitis, some of which may eventually require liver transplantation[72, 73]. Especially patients with severe hepatitis and underlying chronic liver disease have a poor prognosis, although currently several have been treated successfully with ribavirin[74].

Treatment of acute HEV infections in immune-competent individuals mainly consists of symptomatic treatment in combination with the use of antiviral

moieties like ribavirin[75]. This thesis focuses predominantly on the immune-compromised individuals infected with HEV. Treatment of HEV infection after transplantation includes reduction of immunosuppressive therapy and treatment with anti-viral agents, of which both safety and efficacy have been studied for other infections than hepatitis E virus. No randomised controlled studies have been conducted to evaluate the efficacy of these antivirals for hepatitis E virus infection, due to the relatively small number of chronically infected patients in need of treatment.

The first choice of intervention, reducing the dose of immunosuppressive drugs targeting T cells can lead to HEV clearance in up to one third of patients[71, 76]. Non-specific antiviral drugs targeting the immune system, like Peg-IFN-α-2a/b and oral ribavirin have been successfully used to treat HEV infection in liver- and kidney transplant recipients as well as in a HIV infected patient [27,39 59-61].
Tapering immunosuppressive drugs or treatment with Peg-IFN-α-2a/b is not always possible or desirable due to the high risk of transplant rejection, which may lead to chronic allograft dysfunction and death. For these patients treatment with ribavirin can be considered. Rapid clearance of HEV RNA in plasma (within three months) with normalizing ALT levels are observed after start of ribavirin therapy and a sustained viral load reduction can be induced in patients with chronic HEV infection[77-79]. The optimal daily dose and duration of ribavirin treatment is still largely unknown. In case reports and small case series, sustained response has been described with daily dosages between 200 mg and 800 mg[80] leading to undetectable HEV RNA in the faeces.

Currently there are no more specific antiviral drugs available targeting well defined hepatitis E virus replication steps, like nucleoside analogues in hepatitis B virus treatment or protease inhibitors in hepatitis C infected patients.

HEPATITIS E PREVENTION STRATEGIES

Currently, no specific preventive strategies other than immunization - currently only available in China – are practiced. In general, no surveillance efforts, nor efforts to raise knowledge or awareness among the public are currently in place. Immune-compromised individuals do get dietary advice – not to eat raw meat products – but the advice is not focused specifically on prevention of HEV infection.

Since hepatitis E virus cannot efficiently be cultured, future vaccine development strategies will most likely have to rely on recombinant technologies. In the past decade, many candidate hepatitis E vaccines have been developed, but only two of these were tested throughout phase II-IV clinical trials. Both vaccines are based on genotype 1 (recombinant) ORF2 antigens, are alum adjuvant and are currently used in a regimen of three intra muscular doses at 0, 1 and 6 months. Although human HEV's belong to four genotypes, they are considered to be one serotype, and therefore these vaccines may be expected to be effective to all four genotypes[81-83].

One HEV vaccine candidate is based on a 56kDa recombinant ORF2 protein, produced in a baculovirus vector based expression system in SF2 insect cells. This vaccine has been developed and tested, in a phase I and later a double blinded placebo controlled phase II clinical trial sponsored by GlaxoSmithKline and the United States army[84]. This vaccine showed to be effective in 95% of the –mainly- healthy young men in Nepal. Though, the manufacturer considered the production of this vaccine not cost-effective and clinical trials were stopped.

The second HEV vaccine is based on a short ORF2 peptide, HEV239 (Figure 4) aa368-606, produced in a bacterial (*Escherichia coli*) expression system[85]. The peptide forms dimers and aggregates into virus like particles, which is imperative to induce neutralizing antibodies. It has been shown to induce protective immunity in primates against gt 1 and gt 4 infections[85]. The safety of the vaccine was evaluated in a phase I clinical trial, and showed the vaccine was well tolerated with no serious side-effects. In a double-blind randomized controlled phase II clinical trial a higher (~100%) antibody response and a 89% protective efficacy was achieved after a three dose immunization schedule rather than a two-dose schedule[86]. Later, a large-scale phase III clinical trials confirmed the efficacy data, and showed the vaccine can be used for rapid control measurements in case of an emerging epidemic, since 100% of the participants had antibody responses lasting for at least 5 months after the first dose[87]. After the completion of this phase III clinical trial, the HEV239 vaccine was licensed for the Chinese market in 2011 and launched in 2012 under the name 'Hecolin' by Xiamen Innovax, Xiamen, China. The vaccine is currently not registered in other countries. The phase IV was recently published and is discussed in chapter 5, the summarizing discussion.

OUTLINE OF THIS THESIS

This thesis focuses on the diagnosis and clinical features of HEV infections in immune-compromised patients in the Netherlands and the establishment of an animal model for this disease.
As described in chapter 2, we first assessed commercially available ELISAs, the most basic diagnostic assay for detection of HEV specific antibodies, to reliably study difference of the antibody kinetics in immune-compromised compared to immune competent individuals.
Chapter 3 describes the identification of specific immune-compromised patient groups suffering from hepatitis E and the related clinical sequelae: chapter 3.1 focuses on solid organ transplant recipients, chapter 3.2 on heart transplant patients and chapter 3.3. describes the identification allogeneic haemopoietic stem cell transplantation patients (allo HSCT), as a newly identified immune-compromised patient group with HEV infections.
Chapter 4 describes an *in vitro* method and an *in vivo* model for virus propagation. The *in vivo* model, based on the use of chimeric mice, can also be used as a model to study the pathogenesis of a chronic HEV infection in an immune-compromised host.
Finally, in chapter 5 the findings of these studies are summarized and discussed, also in the light of data generated by other recently conducted studies.

Chapter 2

Diagnostic performance of selected commercial HEV IgM and IgG ELISAs for immunocompromised and immunocompetent patients

Suzan D. Pas, Roel H.R.A. Streefkerk, Mark Pronk, Robert A. de Man, Matthias F. Beersma, Albert D.M.E. Osterhaus and Annemiek A. van der Eijk

Journal of Clinical Virology 2013; 58: 629– 634.

ABSTRACT

Background

Hepatitis E virus (HEV) genotype 3 is recognized as an emerging pathogen in industrialized countries. The currently commercially available HEV specific enzyme linked immunosorbent assays (ELISAs) are primarily designed for the detection of antibodies against genotypes 1 (Birma) and 2 (Mexico) and may not sensitively detect HEV genotype 3 or 4.

Objectives

To evaluated the analytical and clinical performances of eight commercially available HEV serum antibody IgM and IgG specific ELISAs for genotype 1 and 3 HEV infections in a clinical setting. To study the antibody responses against HEV of immunocompromised versus immunocompetent patient groups.

Study design

Analytical performance, diagnostic sensitivity and specificity was assessed using well-defined reference samples and samples from patients with PCR-confirmed HEV infection (n=88) and a specificity panel (n=98).

Results

Limiting dilutions indicated that highest analytical sensitivity in head-to-head comparison was measured for the Mikrogen_new IgG assay. Taking the serum working dilutions of each assay into account, the Wantai IgG assay was the most sensitive assay . ROC analysis showed AUC-values of 0.943, 0.964, 0.969, 0.971, 0.974 and 0.994 for the DSI, Mikrogen_old, MP-diagnostics, Mikrogen_new, Wantai and DiaPro anti-HEV IgM assays, respectively. Highest specificity of currently available assays was found for the IgM Wantai assay (>99%). If anti-HEV IgM and IgG results from each supplier were combined, DSI and Wantai assays were able to detect the highest number of (passed) HEV infections.

Conclusions

Our study shows that current commercial HEV ELISAs can be used to diagnose HEV genotype 3 infection adequately in a clinical setting.

BACKGROUND

Hepatitis E virus (HEV) is a positive sense, single-stranded RNA virus that causes subclinical, acute and chronic infections, characterized by hepatitis, though extra-hepatic manifestations have been described. Four genotypes are known to infect humans (genotype 1-4), of which epidemiology and geographical distribution differs between genotype 1-2 and 3-4. HEV genotype 3 and to lesser extend genotype 4, is recognized as an emerging pathogen in industrialized countries[40, 88] and can cause chronic hepatitis in immunocompromised individuals leading to rapid fibrosis of the liver[89].

HEV infection is diagnosed by laboratory testing, since it's clinical presentation does not differ from other pathogens causing hepatitis. Till this date the virus cannot efficiently be cultured for diagnostic purposes, thus detection of IgM and IgG antibodies and virus RNA are the modes to confirm HEV infection[38].

In immunocompromised patients (e.g. solid organ transplant recipients, hematological patients) antibody production is often delayed[39] and detection of HEV RNA is suggested to diagnose HEV infection in this patient group[90]. In immunocompetent individuals, the narrow window in which HEV RNA can be detected in serum or feces, is confined to the acute phase of the disease (mean 28, range 17-48 days)[40]. Therefore serology is needed to diagnose HEV infection in patients who present themselves after vireamic period.

Though HEV has one serotype, the role of currently commercially available serological assays is questioned in genotype 3 and 4 endemic countries. The current commercial HEV specific enzyme linked immunosorbent assays (ELISAs) have recombinant ORF2/3 antigens coated which are primarily designed for the detection of antibodies against genotypes 1 (Birma) and 2 (Mexico) and may not sensitively detect HEV genotype 3 or 4[41, 90-92]. The sole ELISA assay using genotype 1 and 3 antigens (Mikrogen) was recently introduced in the market.

A few in-house assays have been described[93, 94], but since continuity and robustness of routinely used assays is essential in clinical settings, most laboratories prefer (CE-marked / FDA-approved) commercial assays. The HEV specific immunoblot (Recomblot, Mikrogen) [91, 95] uses the same antigens as the Mikrogen IgM/IgG ELISA and is labor intensive. The knowledge on the performance of current commercially available IgM and IgG ELISAs is limited, specifically if IgM and IgG of the same commercial company is preferably combined in routine work-up.

Available assays differ in the accuracy for detection of an acute or past HEV infection. In addition, previous publications have assessed the performance of either HEV specific IgG [41, 96-99] or IgM [93, 95] solely.

OBJECTIVES

We have evaluated the analytical and diagnostic performance of selected commercially available IgM and IgG ELISAs for the detection of both genotype 1 and 3 HEV infections, using a well-defined serum-panel of PCR confirmed HEV infected patients. Secondly, we studied the HEV antibody responses in immunocompetent and immunocompromised patients.

STUDY DESIGN

Sample collection

The samples used in our retrospective study had been collected in the time period of 2003-2011 during hospitalization and routine visits to our out-patient clinic for clinical assessments. Serum/EDTA-plasma samples have been stored at -20˚C and -80˚C respectively.

Sensitivity panel

To assess the analytical sensitivity we performed a two-fold endpoint titration of a genotype 1 and 3 HEV IgM and IgG antibody positive serum, starting from 1/125 and 1/25 respectively. Presence of HEV antibodies of these secondary standards was confirmed by the -previous- routinely used MP-Diagnostics ELISA and HEV RNA was detected by HEV RT-PCR. Additionally, for IgG a two-fold endpoint titration (starting from 1/25) was performed using the WHO reference reagent for HEV antibody, (human serum NIBSC code: 95/584) of which the antigenic trait is unknown[100]. Lower limit of detection (LLOD) was calculated for each anti-HEV IgG ELISA using the WHO IS, taking the working dilution of each assay into account, as described before[41].

To select samples for the diagnostic sensitivity panel, a LIMS database search was performed for HEV RNA positivity, HEV genotype, immune status, sequential sample availability and clinical information. Eighty-eight samples were selected from 17 immunocompromised, 15 immunocompetent and 4 patients with an unknown immune status (total 36), whose HEV infections were confirmed by real-time RT-PCR. Time frame of infection was determined with reference to clinical symptoms and retrospective HEV PCR testing (Table 1).

Table 1 Sample distribution of diagnostic sensitivity panel

Time of drawl (t=)	Samples (*n*=)	Patients (*n*=)	Samples per immune status[a] (*n*=)			Samples per genotype (*n*=)		
			IC	ICT	Unkn	geno1	geno3	Unkn
Prior to infection	12	12	12	0	0	0	9	3
< 6 wks	34b	31	16	14	4b	7	18b	9
> 6 wks < 6 mos	22	19	15	4	3	0	16	6
>6 mos	20	16	16	3	1	1	17	2

[a] ICT Immunocompetent, IC=immunocompromised, Unkn = unknown
[b] one sample was excluded in the Mikrogen_new assay due to sample volume.

Specificity panel

To assess the assay's specificity, a serum/EDTA-plasma sample panel was constituted of acute Epstein-Barr virus (EBV) infection (n=10), human cytomegalovirus (CMV) infection (n=10), B19 virus infection (n=10), hepatitis A virus (HAV) (n=10), hepatitis B virus (HBV) (n=10), hepatitis C virus (HCV) infections (n=10) and healthy blood donors (n=28). Additionally 10 samples were selected from 10 HEV infected transplant recipients prior to their HEV infection.

Selection of immunoassays for HEV antibody detection

For detection of both anti-HEV IgM and IgG in serum or EDTA-plasma samples, eight commercially available HEV ELISAs were selected on basis of prior publications[41, 93], common use in Dutch laboratories and availability. IgM and IgG ELISAs were selected from MP-diagnostics, (IgM v3.0, MP Biomedicals, Singapore, former Genelabs (GL)), Dia.Pro (Milano, Italy), DRG (Marburg, Germany), DSI[93] (RPC Diagnostic systems, Novgorod, Russia), Diacheck (MP-products, the Netherlands), Wantai Biological Pharmacy (PE2-assay, Beijing, China) and Mikrogen (recomWell Neuried, Germany). From Mikrogen, two versions recomWell anti-HEV IgM assays were selected, one available in The Netherlands up until Feb. 2011, and the 'new' version available from Nov 2012 in the Netherlands.

All HEV IgM and IgG ELISAs indirectly detected HEV antibodies using synthetic ORF2 and 3 peptides coated on a polystyrene plate, except for the HEV-IgM PE2 ELISA from Wantai being an μ-chain capture ELISA[101]. In all ELISAs, genotype 1 and 2 antigens were used, except for the new version RecomWell HEV-IgM assay (Mikrogen), which is coated with genotype 1, 2 and 3 ORF3 antigens.

ELISA procedures were used according to the manufacturer's instructions, positive and blank controls were taken for quality assurance. Signal to cut-off (s/co)-ratios were interpreted according to manufacturer's instructions. Subsequent statistical analysis was performed using IBM SPSS statistics software v.20.

HEV-RNA detection and sequence analysis

The internally controlled quantitative real-time RT-PCR amplified a conserved ORF3 region of 77bp as described before [89, 90]. For phylogenetic analyses, ORF1 RdRp (nt 4254–4560) sequences of 321 bp were generated on a ABI3130XL using previously described methods[90].

RESULTS

Analytical sensitivity

IgM limiting dilutions indicated that the highest analytical sensitivity among the IgM ELISAs for genotype 1 was achieved by MP-Diagnostics and Wantai assay. For genotype 3 IgM antibody titration, the Wantai assay was the most sensitive assay (Table 2). Remarkably, a wide variety of s/co -ratio's was observed of which the IgM and IgG Wantai assay stood out (s/co-ratios of 18 as upper limit of detection).

IgG limiting dilutions indicated that the highest analytical sensitivity in head-to-head comparison (titration curves) was measured for the Mikrogen_new assay. Though, the Wantai assay had an equal sensitivity for genotype 1 (Table 2). Remarkably, if the LLOD was calculated taken the serum working dilutions of each assay into account, the Wantai anti-HEV IgG assay was the most sensitive assay with 0.69 IU/ml (Table 2). For all samples, the IgM and IgG Diacheck assays had the lowest sensitivity and were therefore excluded for further validation. Additionally, both Dia.Pro and DRG proved to be the exact same assay, though from two different manufacturers and we therefore excluded the most expensive assay (DRG) from further validation.

Table 2 Performance of IgM and IgG specific ELISAs

	analytical sensitivity (titers)						clinical performance IgM		
	IgM (titers)		IgG (titers)			LLOD* IgG (IU/ml)	ROC* analysis	Sens.	Spec.
	geno1	geno3	geno1	geno3	WHO*	WHO*	AUC (95%CI)		
Mikrogen_old	4000	250	6400	800	1600	6.31	0.964 (0.940-0.989)	52%	>99%
Mikrogen_new	32000	16000	>12800	3200	3200	3.16	0.971 (0.938-1.000)	74%	99%
MP-Diagnostics	>64000	4000	3200	100	800	2.63	0.969 (0.948-0.991)	74%	84%
DSI	8000	4000	3200	800	800	1.25	0.943 (0.900-0.986)	71%	90%
Dia.Pro	32000	32000	6400	100	800	2.63	0.994 (0.982-1.000)	81%	98%
Wantai	>64000	>64000	>12800	1600	1600	0.69	0.974 (0.941-1.000)	75%	>99%
DRG	32000	32000	6400	100	800	2.63	excluded		
Diacheck	1000	125	3200	100	400	5.25			

* WHO reference sample, lower limit of detection (LLOD) taking the serum work dilution of each assay into account, receiver operator curve (ROC), area under the curve value (AUC), confidence interval (CI), sensitivity (sens.), specificity (spec.)

Specificity anti-HEV IgM ELISAs

The specificity panel was assessed only for IgM, since no gold standard for IgG is available. From the specificity panel (n=98), 16 samples were reactive in the IgM MP-Diagnostics assay, 10 samples in the IgM DSI assay, 2 samples in the IgM Dia.Pro assay, 1 sample in the IgM Mikrogen_new assay and none in the IgM Mikrogen_old and Wantai assay. HEV-RNA could not be detected in these reactive sera by sensitive real-time RT-PCR assay. The boxplot (Figure 1), shows a high variety of aspecifc reactions among the different IgM assays in specific subpanels of acute infections. Two IgM assays (DSI and MP-Diagnostics) showed aspecific reactions with sera from both acute CMV (n=6 and 5, respectively) and HAV infections (n=2 and 3, respectively). Remarkably, the DSI IgM ELISA, had a median s/co-ratio above the cutoff of the assay in case of acute CMV infections. Noteworthy are the low s/co-ratio's in all groups of the specificity panel of the Wantai assay, since this assay had the highest s/co-ratio's in HEV-IgM/IgG positive sera. The overall specificity was calculated (Table 2), resulting in the highest score for IgM Mikrogen_old (>99%) and IgM Wantai assay (>99%).

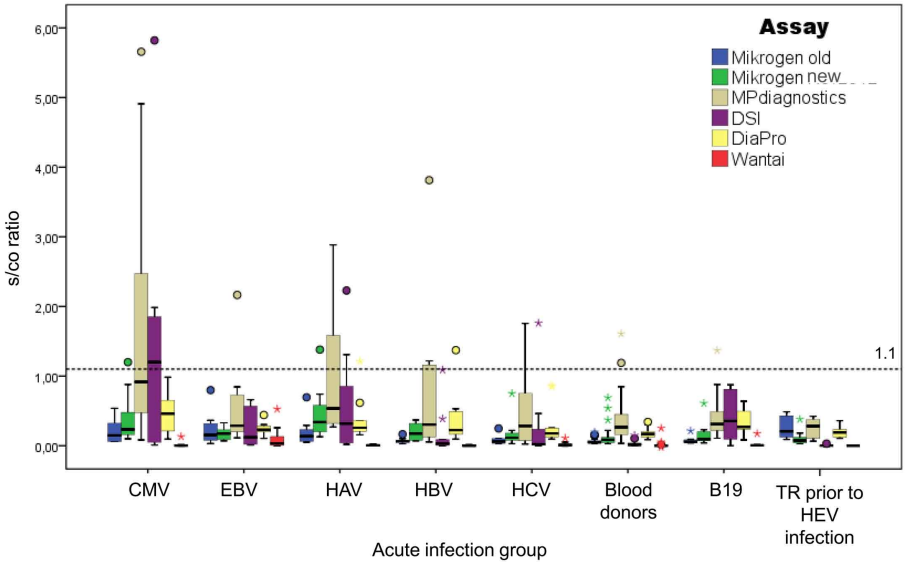

Figure 1 Specificity of selected anti-HEV IgM ELISAs. Boxplot showing the mean, interquartile ranges, outliers (o) and extremes (*) of the signal to cut-off (s/co) ratios of six HEV-IgM detecting ELISAs in patients with diagnosed acute infections of CMV (n=10), EBV (n=10), HAV (n=10, n=9 for Mikrogen_new assay), HBV (n=10), HCV (n=10), B19 (n=10), healthy blood donors (n=28) and samples of transplant recipients (TR) before HEV infection (n=10). Colors are indicated in the legend (o Mikrogen_old, o Mikrogen_new, o MP-diagnostics, o DSI, o DiaPro, o Wantai)

Diagnostic sensitivity of the anti-HEV IgM ELISAs

To assess the clinical accuracy of de IgM ELISAs, the diagnostic sensitivity panel was processed using six IgM ELISAs. Cohen's kappa (κ) concordance was calculated, with 'intermediate results (s/co-ratio's interpreted according to manufacturer's instructions) both considered as reactive or non-reactive, which yielded higher or equal concordance levels for the assays using 'intermediate' results assigned as non-reactive (Table 3). The κ-levels ranged between 0.562 and 0.948 and the highest κ-levels among the IgM ELISAs were the Dia.PRO/Wantai IgM assays (κ=0.900) and the Mikrogen_new/Wantai IgM assays (κ=0.948).

Furthermore, receiver operator curve (ROC) analysis resulted in highest area under the curve of 0.971, 0.974 and 0.994 for the Mikrogen_new, Wantai and DiaPro HEV IgM assays, respectively (Table 2).

The overall diagnostic sensitivity ranged from 52% for the Mikrogen_old IgM assay to 81% for the IgM Dia.PRO assay (Table 2). If only acute samples (<6wks after infection, n=34) were taken into account, the diagnostic sensitivity was 65% for Mikrogen_old, 73% for Mikrogen new, 74% for MP diagnostics, DSI and Wantai and 79% for Dia.Pro assays.

Table 3 Cohen's Kappa concordance of anti-HEV IgM and IgG specific ELISAs

		anti-HEV IgM					
		Mikrogen_old	Mikrogen_new	MP-Diagnostics	DSI	Dia.Pro	Wantai
anti-HEV IgG	Mikrogen_old	-	0.744	0.562	0.642	0.653	0.721
	Mikrogen_new	0.739	-	0.743	0.810	0.873	0.948
	MP-Diagnostics	0.643	0.559	-	0.738	0.726	0.723
	DSI	0.783	0.854	0.621	-	0.792	0.763
	Dia.Pro	0.678	0.667	0.813	0.653	-	0.900
	Wantai	0.730	0.893	0.530	0.892	0.610	-

Performance of combined anti-HEV IgM and IgG ELISAs

Since in most clinical laboratories IgM and IgG ELISAs of one commercial provider are combined in routine work-up, we assessed the combined results of both anti-HEV IgM and IgG ELISAs of one provider to diagnose a HEV infection at different time points in the infection. None of the assays were able to detect anti-HEV IgM nor IgG in 9 of 78 samples, though HEV RNA could be detected (median viral load 2.35 log10 IU/ml, range 1.41-7.09). These nine samples belonged to seven immunocompromised transplantation recipients, of which six samples were drawn <6 weeks after infection and two between 6 weeks and 6 months after infection. Anti-HEV IgM and/or IgG antibodies (IgM/IgG ratio) were detected in 51 (40/40), 61 (57/33), 63 (62/39) and 66 (56/56) samples for Mikrogen_old, MP-Diagnostics, Dia.PRO assay and Mikrogen_new assay respectively, whereas both DSI and Wantai IgM and IgG assays combined could detect HEV specific antibodies in 67 of 78 samples. For these two assays IgM/IgG nominator of positive samples was 55/50 and 58/57 samples respectively.

Antibody kinetics of genotype 1 and 3 in the immunocompetent and immunocompromised

To gain insight in the antibody kinetics in groups with different immune status and detected responses to the two assessed genotypes, the s/co-ratios of these different panels were plotted (Figure 2 and Figure 3). Figure 2 shows a difference in magnitude of the immune responses in the compared groups. However anti-HEV IgM could be detected in only in 7/16 immunocompromised patients and 18/18 immunocompetent patients in the acute phase (<6wks) of the infection, indicating a delayed immune response in the immunocompromised group. The immunocompetent group shows a classic pattern of quickly rising IgM levels immediately after infection, which declines after 6 weeks and becomes

undetectable after 6 months (Figure 2, lower left panel). In contrast to the immunocompromised group (of which 50% were chronically infected), having low IgM levels immediately after HEV infection, which rise during the course of infection. Furthermore, Figure 3 shows that genotype 3 IgM antibodies can efficiently be detected in assays which have genotype 1 and 2 antigens coated.
The difference between immunocompromised and immunocompetent groups in IgG antibody kinetics is less apparent than for IgM, both showing seemingly steady IgG antibody levels after 6 weeks of infection (Figure 2, right panels). However, only two assays (Mikrogen_new and Wantai) were able to detect IgG in all samples of the immunocompetent group (n=4) six months after infection. Also for IgG, genotype 3 antibodies were detected using genotype 1 and 2 coated ELISAs.

DISCUSSION

Autochthonous HEV infection, caused by genotype 3, is recognized as an emerging infectious disease in industrialized countries. Only limited data are available on the diagnostic performance of commercial IgM and IgG ELISAs or combination of these two and the sensitivity of ELISAs coated with genotype 1 and 2 antigens is questioned for the detection of genotype 3 and 4. Our study gives more insight in diagnostic performance and antibody kinetics of commercial anti-HEV IgM and IgG assays of immunocompromised and immunocompetent patients with genotype 1 and 3 HEV infections.
The results of this study show a variety in analytical sensitivity among the tested assays for both IgM and IgG antibodies and if used on clinical specimens also varying clinical specificity ranging from 84 – more than 99% and sensitivity ranging from 52-79%. If the IgM and IgG results from each commercial supplier were combined to assess the diagnostic value, only DSI and Wantai assays were able to identify HEV specific antibodies in 86% of samples. These data should be interpreted for each setting separately, depending on the objective for which the ELISAs is used. In clinical practice, IgM is the most valuable serological tool to diagnose an acute HEV infection. Comparing the currently available assays, the Wantai assay had the best specificity (>99%), most discriminative s/co-ratios and comparable diagnostic sensitivity. For public health studies in genotype 3 endemic areas, it should be taken into account that the Mikrogen_new assay had a better analytical sensitivity for IgG if head-to-head compared to other assays. However, taking the working dilution of this assay (1:101) and the Wantai

assay (1:11) into account, Wantai had the lowest LLOD (0.69IU/ml). Furthermore, specificity could not be assessed for IgG and we did not include samples with a long (>5 years) follow up period and can therefore not draw any conclusions on performance of the selected assays over longer periods of times.

It is known that antigenicity of the pE2 peptide (aa394-606) used in the Wantai assay, is superior to other shorter E2 peptides, because of the stabilizing effect an 60 amino acid extension has on the dimer formation of the antigen[27]. The Wantai assay is the only assay which uses an μ-chain capture strategy in the anti-HEV IgM assay. Generally, μ-chain capture ELISAs have higher specificity and sensitivity than indirect ELISAs[102, 103]. Taken together, this may explain the high s/co-ratios seen in the Wantai anti-HEV IgM and IgG assays.

The seemingly moderate diagnostic sensitivity in this study may be explained by the 67.1% of the samples in this sensitivity panel, which were selected from 17 immunocompromised individuals, having HEV-RNA in their peripheral blood, though inferior antibody levels than immunocompetent individuals. In 11.5% of the patients none of the assays could detect any antibody responses.

Furthermore, antibody kinetics directed against genotype 1 and 3 infections in groups with different immune status confirmed findings of previous studies[95, 99], concluding there are no sensitivity problems in the detection of genotype 3 using ELISA assays coated with only genotype 1 and 2 antigens. Though the diagnostic sensitivity panels used in our study indicate the different antibody kinetics among immunocompromised compared to immunocompetent group, the numbers used were relatively small and need to be assessed eventually in larger panels to gain statistical significance. A second limitation of our study is the absence of HEV genotype 2 and 4 samples, which were not available for this study.

Collectively, though there is a wide variety among the selected assays, our data show that the HEV ELISAs can be used to diagnose both HEV genotype 1 and 3 infections in a clinical setting. Considering the high concordance and specificity of the best performing assays and the availability of HEV RNA assays, there's no longer a need to perform serologically confirmatory testing in diagnostic settings.

ETHICAL APPROVAL

This study was approved by the hospital medical ethical committee (MEC-2011-277).

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Chapter 3

Clinical implications of hepatitis E

Chapter 3.1

Hepatitis E virus infection in solid organ transplant recipients in the Netherlands

Suzan D. Pas, Rob A. de Man, Claudia Mulders, Aggie H.M.M. Balk, Peter Th.W van Hal, Willem Weimar, Marion P.G. Koopmans, Albert D.M.E. Osterhaus and Annemiek A. van der Eijk

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ABSTRACT

The recent increase in reports of hepatitis E virus (HEV) infections in solid organ transplant (SOT) recipients prompted us to screen all living heart- (HTX), lung- (LungTX), liver- (LTX) and kidney- (KTX) transplant recipients (n=1197) attending the out-patient clinic of the largest transplantation centre in The Netherlands. In each SOT-category, HEV infections were found. In twelve patients (1%) HEV infection was identified by RT-PCR, ten of them had developed symptomatic chronic HEV infection. Phylogenetic analyses of the ORF1 sequences of the HEVs involved, classified all within genotype 3, without indications for a common source. Retrospective analysis of samples from the confirmed cases revealed that HEV RNA was detected on average 64 days prior to HEV specific IgM. This is the first systematic survey among all SOT recipients, confirming that they are at risk to develop chronic HEV infection, and stressing the importance of HEV RNA screening in SOT recipients.

INTRODUCTION

Hepatitis E virus (HEV) may cause acute or chronic infection in humans, characterized by hepatitis and gastro-enteritis. HEV is shed through stool of infected individuals, and most commonly transmitted by fecally contaminated water. In addition to this, food-borne and blood-borne transmission have been documented, and direct zoonotic transmission has been suggested. Direct person-to-person transmission is thought to be rare. Hepatitis E viruses are small (24-34 nm) non-enveloped viruses, with a single stranded, positive sense RNA genome of around 7,500 base pairs and three partially overlapping open reading frames (ORF 1-3)[104]. Four genotypes (1-4) have been described in humans. Genotype 1 is responsible for large water-borne HEV outbreaks in endemic non-industrialized countries. In industrialized countries HEV genotype 3 predominantly infects pigs, wild boars and deer but also humans and is recognized as a zoönotic agent. However the main modes of transmission of genotype 3 viruses remain to be determined [40, 105]. Prevalence of genotype 3 HEV RNA in pooled grab samples collected at Dutch pig farms was estimated to be about 55%[106] while 6.5% of commercially available porcine livers tested positive for HEV RNA, while the viruses could also be detected in surface waters [52]. This indicates that insufficiently heated pig meat products or environmental exposures may be a source of human infection. As awareness for HEV infection is rising, the numbers of human case reports of HEV infection also increase. Especially the numbers of reports concerning immune-compromised individuals including solid organ transplant (SOT) recipients[71, 107, 108], hematological patients[109] and HIV infected individuals[110] have increased.

Systematic analysis of exposure histories of newly diagnosed genotype 3 HEV cases has demonstrated that HEV is under diagnosed, but failed to provide a clear source for most infections [44, 111]. Recently, HEV was recognized as a cause of chronic hepatitis in immunocompromised patients [112-114]. On the basis of sero-epidemiological studies it has been estimated that 2% of the Dutch population has been infected with HEV[115], whereas symptomatic acute and chronic infections are diagnosed only sporadically. Given the level of exposure of the population, HEV infections in the Netherlands are probably largely under-diagnosed. Since HEV has been reported to cause acute and severe liver disease in SOT recipients[116], we set out to screen all living SOT recipients visiting the out-patient clinic of ErasmusMC for HEV RNA. ErasmusMC is the largest SOT centre in the Netherlands, where 24% of Dutch SOT recipients are transplanted. At the time of testing this SOT study population consisted of 256 heart- (HTX), 53 lung- (LungTx), 300 liver- (LTX), 574 kidney (KTX) and 14 multiple adult SOT recipients. The cross- sectional analysis

conducted in this study was designed to enable us to identify SOT recipients with acute or chronic HEV infection, eligible for treatment of their hepatitis.

MATERIALS AND METHODS

Sample collection

We conducted a cross-sectional analysis of all living adult SOT recipients of whom serum or EDTA-plasma samples were available in the biobank of ErasmusMC. These samples had been collected during routine visits to our out-patient clinic for clinical assessments and have been stored at -20˚C and -80˚C respectively. To select samples, a LIMS database search was performed for last post-transplantation sample availability (preferably EDTA-plasma) within the following arbitrarily chosen timeframes: HTX from 2000 to March 2011, LTX from 2009 to March 2011, KTX from 2009 to March 2011 and LungTx from 2007 to March 2011. Each enrolled subject had signed an informed consent that allowed future testing of archived bio-samples. Additionally, all HEV RNA positive non-SOT recipients represented in our biobank were genotyped and used as reference for phylogenetic analysis. This study was approved by the medical ethical committee of the hospital (MEC approval: MEC-2011-277).

HEV specific antibody detection

For detection of both HEV specific IgM and specific IgG in serum or plasma samples the commercially available HEV ELISA v3.0 (MPdiagnostics, former Genelabs, Singapore) was used according to the manufacturer's instructions. Samples of HEV-RNA positive patients were retrospectively tested during the entire course of infection to study the kinetics of antibody responses (IgM and IgG) and viremia in the confirmed cases.

HEV-RNA detection

All samples were screened for the presence of HEV RNA by a real-time RT-PCR based on Ahn *et al*.[117] and Zhao *et al*.[118] amplifying a conserved ORF3 region of 77bp. Primers were adapted to detect all four genotypes. Briefly, RNA was extracted using MagnaPureLC (Roche Diagnostics, Almere, The Netherlands) and total nucleic acid isolation kit with an input and output volume of 200µl and 100µl, respectively. The extraction was internally controlled by the addition of a known concentration of Phocine Distemper Virus. 20 µl extracted RNA was amplified in a 50µl final volume one-step RT-PCR, containing 12.5 µl 4x TaqMan®

Fast Virus 1-Step Master Mix (Lifetechnologies, Nieuwerkerk a/d Ijssel, The Netherlands), 0.5 ul (1U/ul) Uracil-N-Glycosylase (Lifetechnologies, Nieuwerkerk a/d IJssel, The Netherlands), 30 pmol HEV fwd ('5- CGGTGGTTTCTGGGGTGA-'3) 45 pmol HEV rev ('5-GCRAAGGGRTTGGTTGG-'3), 5 pmol HEV probe ('5-FAM-ATTCTCAGCCCTTCGC-MGB-'3). The internal control was amplified in a separate reaction using TaqMan® Fast Virus 1-Step Master Mix, 0.5 ul (1U/ul) Uracil-N-Glycosylase and primers/probe as described before [119]. Amplification was performed in a LC480 (Roche Applied Science, Almere, The Netherlands) using Fit point analysis module. Quality assurance was performed using the free MedlabQC software. Criterion for a successful RT-PCR run was Ct values of both internal control and positive PCR control should be within 3xSD of mean.

Case definition

A case of HEV infection was defined as a patient with a HEV RNA positive serum or plasma sample and was confirmed either by showing HEV specific serum IgM or IgG antibody or by showing the presence of HEV RNA in sequential serum or plasma samples. Chronic infection was diagnosed by retrospective testing of stored samples of identified cases and was defined as having HEV RNA in serum or plasma for more than 6 months.

Sequence analysis

For phylogenetic analyses ORF1 RdRp (nt 4254–4560) sequences of 307 bp were generated using previously described primer set MJ-C [45]. Briefly, cDNA was prepared in 50µl volume reaction containing 23µl HEV RNA, 20 pmol EAP 4576–4595 5'-AGGGTGCCGGGCTCGCCGGA-3', 1x first strand buffer, 0.1M DTT, 10mM dNTP, 80U RNAsin (Promega, Leiden, The Netherlands), 200 units Superscript III RT (LifeTech, Bleiswijk, The Netherlands) and 5 min 65°C and 1 hour 50°C as thermal profile. Subsequently, an outer PCR reaction was performed in a 9700 PCR machine (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) amplifying 10µl cDNA in a 50µl final PCR mix volume, containing 1x PCR buffer, 5 mM $MgCl_2$, 10 mM dNTP, 2.5 units HotStarTaq DNA Polymerase (Qiagen, Venlo, The Netherlands), 20 pmol ISP 4232–4253 5'-GTATTTCGGCCTGGAGTAAGAC-3' and 20 pmol EAP 4576–4595 5'-AGGGTGCCGGGCTCGCCGGA-3'. Thermal profile of PCR was 15 min 95˚C, 40 cycles of 20"94˚C, 30"60˚C, 45"72˚C and 10' 72˚C. If necessary, a nested PCR was performed using 2 µl outer PCR product, the same reaction conditions and PCR profile as for outer PCR, but ISP 4232–4253 5'-GTATTTCGGCCTGGAGTAAGAC-3' and IAP 4561–4583 5'-TCACCGGAGTGYTTCTTCCAGAA-3' as primers. The amplicon was sequenced

with 5 pmol of the above-mentioned primers. One microliter of the amplicon was sequenced with the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The PCR products were purified using Performa DTR V3 purification plate (Edgebio, Sopachem, Ochten, The Netherlands) and separated on an ABI 3130XL sequencer (Applied Biosystems). The sequence data were analyzed using a Sequence Navigator software sequencer (Applied Biosystems) and SeqMan v9.0 (DNASTAR, Madison,WI). Obtained HEV sequences and GenBank reference sequences [45] were aligned and phylogenetic relationships were calculated using maximum likelihood method, Kimura-2 parameter (Mega5.05) and bootstrap resampling (n =1,000). The sequences of all isolates were deposited into GenBank under accession no. JQ015399- JQ015448.

RESULTS

A total of 1197 SOT recipients were screened: 256 HTX-, 53 LungTX- , 300 LTX-, 574 KTX- and 14 multiple SOT recipients. The latter group consisted of four patients with a kidney-heart transplant, one patient with a kidney-lung transplant and nine patients with a kidney-liver transplant. Twelve patients with HEV infection were identified: five (1.95%) HTX-, one (1.89%) LungTX-, three (1%) LTX-,and one (0.17%) KTX- recipients and two multiple SOTs (14.2%)(1 HTX-KTX and 1 LTX-KTX). In ten cases (83%) the viral hepatitis had progressed into a chronic HEV infection (Table 1). The median age of all the HEV infected cases was 56.9 years (range 19.9-63.5) and 75% (n=9) were male. Immunosuppression at time of first HEV RNA detection was in ten HEV cases prednisolone and tacrolimus based, combined with either mycophenolate mofetil (n=3) or everolimus (n=2). In two cases immunosuppression was a both cyclosporine and prednisolone (n=1) or both mycophenolate mofetil and prednisolone (n=1) based regimen.

Table 1 Overview of HEV in Dutch Solid Organ Transplant recipients

SOT Group*	Recipients included (No.)	confirmed HEV infection		Chronic HEV
		No.	(%)	No.
HTX	256	5	1.95	5
LungTX	53	1	1.89	1
LTX	300	3	1.00	2
KTX	574	3	0.17	1
Multiple SOT†	14	2	14.29	1
Total	1197	12	1.00	10

* SOT solid organ transplant, HTX heart transplant, LTX liver transplant, KTX kidney transplant, † 9 KTX-LTX, 4 KTX-HTX and 1 KTX-LungTx

Pathology

All chronic HEV cases had elevated liver enzyme values, with a median peak ALAT level of 329 U/l (upper limit of normal (ULN) 30 U/L, range 70-909), ASAT level of 189 U/l (ULN 36 U/L, range 56-1016), γ-GT level of 303 U/l (ULN 49 U/L, range 72-1740). Peak bilirubin levels were elevated in 50% of the cases (ULN 16 U/L, median 15 U/l, range 5-100). From nine patients with chronic HEV infection, liver biopsies were available. Inflammatory activity compatible with viral hepatitis was shown in eight of these cases. Other pathological findings were F0-F2 fibrosis, steatosis 1-2 (Brunt classification), cholestasis and presence of Councilmanbodies.

Virological parameters

All twelve confirmed HEV cases were retrospectively tested for HEV RNA, anti-HEV IgM and IgG to characterize the course of their infection in relation to the transplantation date. One HEV infection was traced back to 2003 (LuTX), one to 2008 (KTX), one to 2009 (multiple SOT recipient, KTX-HTX), seven to 2010 (5 HTX, 1 LTX and 1 multiple SOT recipient, KTX-LTX) and two to 2011 (both LTX). Among the twelve confirmed cases, one LTX patient was identified with an acute HEV infection, who apparently had cleared the virus (HEV RNA undetectable) within 6 days. Anti-HEV IgM was detected at the same date of detecting HEV RNA, however anti-HEV IgG was not detected. In a second acute HEV case (multiple SOT, LTX-KTX) neither anti-HEV IgM nor IgG serum antibodies were detected, but HEV RNA was positive (median Ct 38.6 in consecutive monthly EDTA-plasma specimen) for 3.9 months at time of writing this manuscript, and could therefore not yet be assigned as a chronic infection. In all ten chronic HEV cases HEV RNA was detected for at least 6 months, with a median period of 10.8 months (range 6.3 -55.1) and a median peak Ct value in EDTA-plasma of 20.2 (range 16.7-23.6).

The course of infection of two arbitrarily chosen representative cases of chronic HEV infection, one liver (A) and one heart (B) transplant recipient is shown in Figure 1, respectively. Both patients show a rise in ALAT values at the time of HEV RNA positivity in peripheral blood. In patient A, anti-HEV IgM and IgG serum antibodies were detected at the time of HEV RNA positivity, however in patient B HEV RNA was detected prior to anti-HEV IgM serum antibodies. Both fecal samples collected from these patients (indicated with * in Figure 1) were HEV RNA positive, showing that HEV may indeed be found in the feces of infected patients. In fecal samples collected from patients with confirmed chronic HEV

infection (n=7), HEV RNA was detected during viremic stage (median Ct 18,2 range 15.5-28.0). No fecal samples were available from the two acute cases.

To assess the choice of diagnostic techniques for detection of HEV infection in SOT recipients, we studied the kinetics of antibody responses (IgM and IgG) and viremia in the confirmed cases (n=12). The median lag time from HEV RNA positivity to anti-HEV IgM detection was 64 days (range -35 – 842 days). Four patients did have detectable anti-HEV IgM titer at the same day of HEV RNA positivity, in one case anti-HEV IgM was detected 35 days before HEV RNA and in one case no anti-HEV IgM was detected. HEV specific IgG titers, however, were not detected in five of twelve cases. One case showed borderline reactivity in only one sample and in the other six cases HEV specific IgG titers were detectable on average 129 days later than HEV RNA (range 0- 842 days). In two cases anti HEV IgG was detectable at the same time as HEV RNA detection.
The median time between transplantation and first HEV-RNA positive sample of all confirmed HEV cases proved to be from -0.3 to 20.0 years (median 1.99 years).

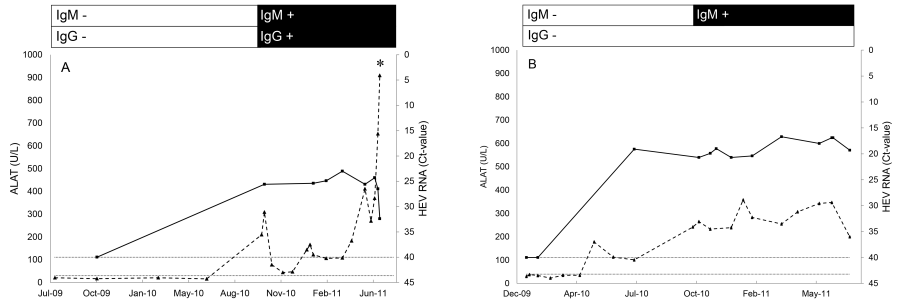

Figure 1 Course of HEV infection in solid organ transplant patients. Plotted are the liver enzyme values, ALAT en HEV RNA Ct-values of a (A) liver transplant patient and a (B) Heart transplant patient prior to and during a chronic HEV infection. Both patient A and B received a tacrolimus based immunosuppressive regimen, patient B also received prednisolone. -■ - HEV RNA (semi-quantative Ct-value), ---▲--- ALAT (U/L), □ Upper Limit of Normal ALAT (=30 U/L); □ Limit of detection HEV RNA (Ct-value = 40). * Feces specimen tested HEV RNA positive.

Phylogenetic analysis

The ORF1b sequences generated from ten cases showed that all these viruses grouped within genotype 3. Since there are no Dutch ORF1b sequences available in GenBank, we generated ORF1b sequences from 40 other acute and chronic Dutch HEV cases as a reference. These samples were found HEV-RNA positive among anti-HEV IgM and/or IgG positive samples from our biobank. Hereafter, the EDTA-plasma or serum-samples of these individuals were retrospectively tested to disclose the time of infection, indicated in the taxon names (Figure 2,

NLyyyy-specimen number). Phylogenetic analyses did reveal indications for neither a common origin nor nosocomial HEV transmission in these SOT recipients.

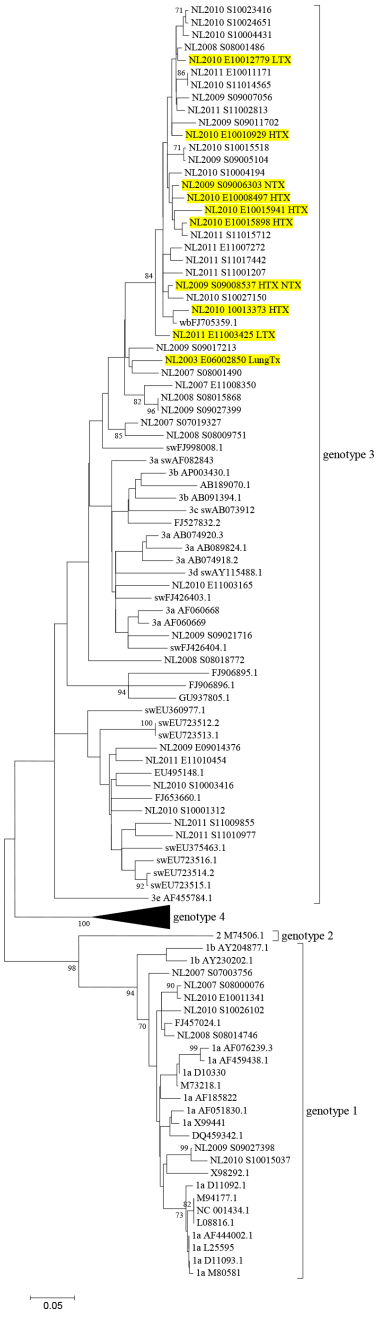

Figure 2 Phylogenetic tree of HEV ORF1 sequences including Dutch cases of acute and chronic HEV infections. Phylogenetic relation of 306 bp ORF1 region was calculated using Maximum likelihood, K2P analysis with bootstrapping (n=1000). Branch lengths are proportional to the evolutionary relationship between the sequences and internodal confidence of >70% is depicted in the tree. HEV sequences of Dutch origin and year of infection are indicated as NLyyyy-isolate number, Genbank accession numbers are no. JQ015399- JQ015448.

DISCUSSION

Recent publications[71, 107, 108, 116] describing HEV infection in SOT recipients, prompted us to perform a retrospective cross sectional survey in all SOT recipients of the largest transplantation centre in the Netherlands. We showed that SOT recipients are indeed at risk of HEV infection. Furthermore, we found that nine (83%) out of twelve chronic cases identified had been treated with a tacrolimus based immunosuppressive regimen. The use of this drug has previously been described as a risk factor for developing chronic HEV infection [71].

The cross-sectional screening by RT-PCR, resulted in the detection of both acute and chronic HEV infections, but could not provide information about previously acquired and cleared HEV infections among the SOT recipients. We screened our SOT population by real-time RT-PCR, for two reasons. First, the immune response of transplant recipients is reduced due to the use of immunosuppressive drugs, and therefore specific antibodies against HEV might not be detectable. Secondly, the currently commercially available HEV specific ELISA's are primarily developed for the detection of antibodies to genotypes 1 (Birma) and 2 (Mexico) and may not sensitively detect HEV genotype 3 or 4 [91].There is only limited information about the validation of HEV genotype 3 serological assays and it has been documented that seroprevalence measured may vary considerably with on the assays used [41, 93, 115]. It should also be noted that detection levels and specificity of HEV RNA among Dutch (unpublished observations) and other European laboratories differ greatly [42]. Therefore standardization with WHO international standards should be encouraged.

Although, at first glance, the observed 1% of actively HEV infected individuals among the SOT recipients may seem low, it should be emphasized that HEV infection can be life threatening in immunocompromised patients. Misdiagnosis of HEV as drug-induced liver injury[120,121] or auto-immune hepatitis infections have been reported and subsequent treatment by raising immune suppression would be counterproductive: in about 30% of the cases temporary reduction of immune suppression resulted in immune mediated control and clearance of HEV [71].
The present study also shows that HEV RNA may be detected on an average 65 and 129 days prior to the appearance of anti-HEV IgM and IgG serum antibodies, respectively. Therefore, in SOT recipients with elevated liver enzyme values (ALAT or ASAT), the diagnosis of HEV infection should be considered and confirmed by HEV RNA detection in addition to other infections causing hepatitis.

In conclusion, this is the first systematic survey of HEV infections among all SOT recipients in a major transplant center, which shows that they indeed are at risk for acquiring chronic HEV infection. Given the serious consequences of chronic HEV infection in immunocompromised individuals, systematic HEV RNA screening of SOT recipients should be implemented, as this life threatening condition may be treated successfully.

ACKNOWLEDGEMENTS

We thank Roel Streefkerk, Mark Pronk, Mark Verbeek, Manon Briede and Sevgi Deniz for technical assistance and Hans Kruining for his help with LIMS database searches.

Chapter 3.2

Clinical implications of chronic hepatitis E virus infection in heart transplant recipients

Suzan D. Pas*, Ludi Koning*, Robert A. de Man, Aggie H.M.M. Balk, Robert J. de Knegt, Fibo J. ten Kate, Albert D.M.E. Osterhaus and Annemiek A. van der Eijk

*Both authors contributed equally

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ABSTRACT

Background & Aims: Recent reports have shown that hepatitis E virus (HEV) infection can become chronic in solid organ transplant recipients, but few studies have systematically investigated the clinical consequences of this chronic HEV infection in solid organ transplant (SOT) recipients.

Methods: We describe an in depth study of six chronic HEV infected heart transplant recipients to gain more insight in clinical, biochemical and virological presentation .

Results: In six patients (2,3%) chronic HEV infection, genotype 3, was identified. Immunosuppression in these patients was tacrolimus based, combined with either everolimus or prednisolone and/or mycophenolate mofetil. Median follow-up after case detection was 26 months (range 21-40 months). All chronic HEV cases had elevated liver enzyme values. IgM antibodies at presentation were positive in two out of six (33%) patients. Liver histology showed in four out of six (67%) patients developed advanced fibrosis within two years after infection. One patient spontaneously cleared the HEV infection, one after dose reduction of immunosuppressive therapy and three during ribavirin therapy. One patient has not managed to clear the virus yet and is still on ribavirin therapy.

Conclusion: chronic HEV infection in HTX recipients may lead to rapid fibrosis of the liver. We advise additional HEV RNA screening in solid organ transplant recipients with elevated liver enzymes, since antibody production is often delayed as shown in these patients. Dose reduction of immunosuppressive therapy should be the first intervention strategy to achieve viral clearance in chronic HEV infected immunocompromised patients. Ribavirin treatment should be considered in cases of chronic HEV.

INTRODUCTION

Hepatitis E virus is a non-enveloped virus with a single-stranded, positive sense RNA genome of approximately 7,500 base pairs and three partially overlapping open reading frames (ORF 1-3) [15]. There are four mammalian genotypes prevalent: genotypes 1 and 2, endemic in developing countries causing waterborne outbreaks and genotypes 3 and 4, seen in sporadic cases in industrialized countries and thought to be zoonotic of origin[15, 105].

In the Netherlands, the prevalence of genotype 3 hepatitis E virus in fecal tanks on pig farms is estimated to be about 55% [51], while HEV RNA was found in 6.5% of commercial porcine livers [52], suggesting a role for undercooked pig meat in pig-to-human infection. Contributing to this theory is the homology between strains detected in pigs that were also found in Dutch patients of up to 100% [44, 106]. Close nucleotide identity to strains isolated in Dutch pigs was also found in HEV infected patients in Germany and Scandinavia[112, 122], which implies a shared distribution of genotype 3 hepatitis E virus in these countries and possibly the rest of Western Europe.

While hepatitis E virus has always been considered to cause solely acute infection, the last few years reports on persistent chronic infection with genotype 3 in immunocompromised patients, mostly with solid organ transplantation, have been published [69, 90, 107, 112, 122-124]. Moreover, chronic HEV infection in these patients can lead to rapid fibrosis and even cirrhosis [112, 113, 122]. Therefore diagnosis of chronic HEV infection in solid organ transplant recipients is vital, to start early intervention and prevent irreversible liver damage. Though some case studies have addressed intervention with ribavirin or peginterferon-alpha treatment, there is no current guideline or standardized treatment protocol available [80, 124-130]. Few studies have systematically investigated the clinical consequences of this chronic HEV infection in solid organ transplant (SOT) recipients. In a previous study, we found a HEV point prevalence of 1% in 1200 SOT recipients [90]. Half of HEV infected patients were heart transplant (HTX) recipients. The aim of the current study was therefore to investigate the clinical presentation of HEV infection in heart transplant (HTX) recipients and management options including antiviral treatment.

MATERIALS AND METHODS

Case definition and sample collection

In previous study we identified one patient with a transient viremia and six chronically HEV infected HTX recipients in a timeframe of 2000-2011 [90]. A case of HEV infection was defined as a patient with an HEV RNA positive serum or EDTA-plasma sample and was confirmed either by showing HEV specific serum IgM or IgG antibody or by showing the presence of HEV RNA in sequential serum or plasma samples. Chronic infection was defined as having HEV RNA in serum or EDTA-plasma for more than 6 months and diagnosed by retrospective testing of stored samples. These samples were collected during routine visits to our outpatient clinic for clinical assessments had been stored at -20°C (serum) and -80°C (EDTA-plasma or feces). To verify excretion of HEV RNA via stool, available fecal samples were screened by RT-PCR. Additionally, spouses of identified cases were asked to donate a serum sample for serological screening of HEV specific antibodies. Each enrolled subject had consented in future testing of archived bio-samples. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the local medical ethical committee (MEC approval: MEC-2011-277).

HEV specific antibody detection

For both HEV specific IgM and HEV specific IgG detection in serum or EDTA plasma samples the commercially available enzyme-linked immunosorbent assay (ELISA) (Wantai, Beijing, China) was used according to the manufacturer's instructions.

HEV-RNA detection

All samples were screened for the presence of HEV RNA by an internally controlled quantitative real-time RT-PCR, described previously [90]. The RT-PCR had a lower limit of detection (95% hit rate) of 143 IU/ml as determined by the 1st WHO stardard for HEV RNA NAT-Based assays (6329/10, Paul Ehrlich Institute, Germany).

Sequence analysis

For phylogenetic analyses ORF1 RdRp (nt 4254–4560) sequences of 306 bp were generated using previously described primer set MJ-C [45]. Detailed methods have been described previously [90]. The sequences of all isolates were deposited into GenBank under accession no. JQ15418, JQ15423-JQ15425 and JQ15427-JQ15428.

Liver biopsy

Liver biopsies were performed using either a 14-gauge needle or an 18-gauge needle in combination with a plugged biopsy. All samples were fixed with formalin, embedded in paraffin and subsequently stained. Staining included: Hematoxylin and Eosin (HE), Periodic acid-Schiff (PAS), PAS-diastase (PAS-D), sirus red, copper, iron, reticulin and CK7. All biopsies were evaluated by the same pathologist (FJtK). Necroinflammatory activity was scored using the Histology Activity Index [131], while the grade of steatosis and iron deposition were classified according to respectively Brunt *et al*. [132] and Brunt *et al*. [133].

RESULTS

Patients

A total of 1200 SOT recipients were screened previously, of which 263 were HTX recipients [90] (Figure 1). Out of all HTX recipients, four were multiple SOT recipients with a kidney-heart transplant. All HEV PCR positive cases were retrospectively tested for HEV RNA, anti-HEV IgM and IgG to characterize the course of the infection in relation to the date of transplantation.

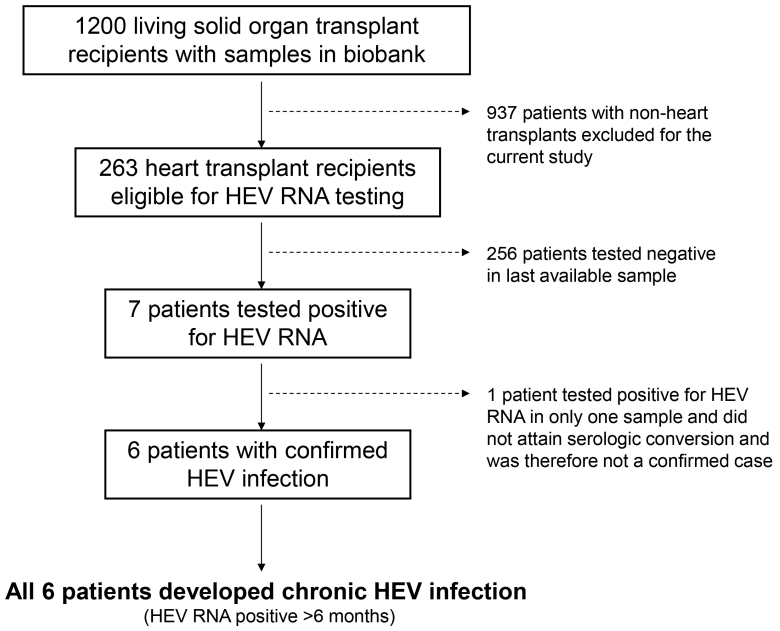

Figure 1 Flow chart of patient selection

In seven (2.7%) patients HEV RNA was detectable in at least one sample. HEV infection could be confirmed in six patients: five heart transplant (HTX) recipients and one kidney-heart transplant (HTX KTX) recipient. In one patient, HEV RNA was only detected in a single sample and could not be confirmed by either detectable HEV RNA or HEV specific IgG or IgM antibodies in any previous or subsequent samples available; therefore this patient did not fulfill the case definition. Moreover, no elevation of liver enzymes was documented in this patient.

All six confirmed cases developed a chronic HEV infection (HEV RNA detectable for at least six months). The median age of all HEV infected patients at the time of infection was 50.0 years (range 38.3-62.4) and 83% (n=5) were male. Median time from transplantation to HEV infection was 7.5 years (range 1-20 years). In the samples studied, all chronic patients were infected after 2008. All six chronic HEV infected patients received tacrolimus based immunosuppression (Table 1).

Table 1 Baseline* characteristics of six HTX recipients with chronic HEV infection

	Age (years)	Gender	Year of trans-plantation	Year of first HEV RNA + sample	Time HTX to infection (years)	Immuno-suppressive therapy	IgM	IgG
1	51.3	M	2008	2010	2	P/T/MMF	+	+
2	55.7	M	1997	2010	13	P/T	-	-
3	47.2	F	2009	2010	1	P/T	+	-
4	62.4	M	2008	2010	2	P/T	-	-
5	49.3	M	HTX: 1996 KTX: 2008	2009	13	E/T	-	-
6	38.3	M	1990	2010	20	P/T	-	-

*Characteristics at the time of first HEV RNA positive sample, M=male, F=female, HTX=heart transplant, KTX=kidney transplant, P=prednisolone, T=tacrolimus, MMF=mycophenolate mofetil, E=everolimus, C=cyclosporin

Virological parameters

All chronic infected patients produced HEV specific IgM antibodies at some time point after HEV RNA became detectable in serum, though in only two patients (33%) IgM antibodies were positive at time of first HEV RNA positive sample. The median time from the first HEV RNA positive sample to IgM seroconversion was 122 days (range 0-301 days). The median time to IgG seroconversion

varied widely from 127 days before seroconversion of IgM to 475 days after seroconversion of IgM.
Fecal HEV shedding was found in all chronic HEV infected patients during viremia. Spouses of patients were tested for HEV infection. None of them had detectable HEV RNA or experienced anti-HEV IgM or IgG seroconversion in their serum.

Phylogenetic analysis
The ORF1b sequences generated from the six chronic cases showed that all virus isolates grouped within genotype 3. Both HEV genotype 3 genbank sequences (accession numbers are taxon names, Figure 2) and HEV ORF1b sequences published previously [90] (accession no. JQ15401, JQ15406-JQ15417, JQ15419-JQ15422, JQ15426 and JQ15429-JQ15448) as reference. The latter samples were retrospectively tested to disclose the year of infection, indicated in the taxon names (Figure 2, NLyyyy-specimen number). Phylogenetic analyses did neither reveal indications for a common origin, nor for nosocomial HEV transmission.

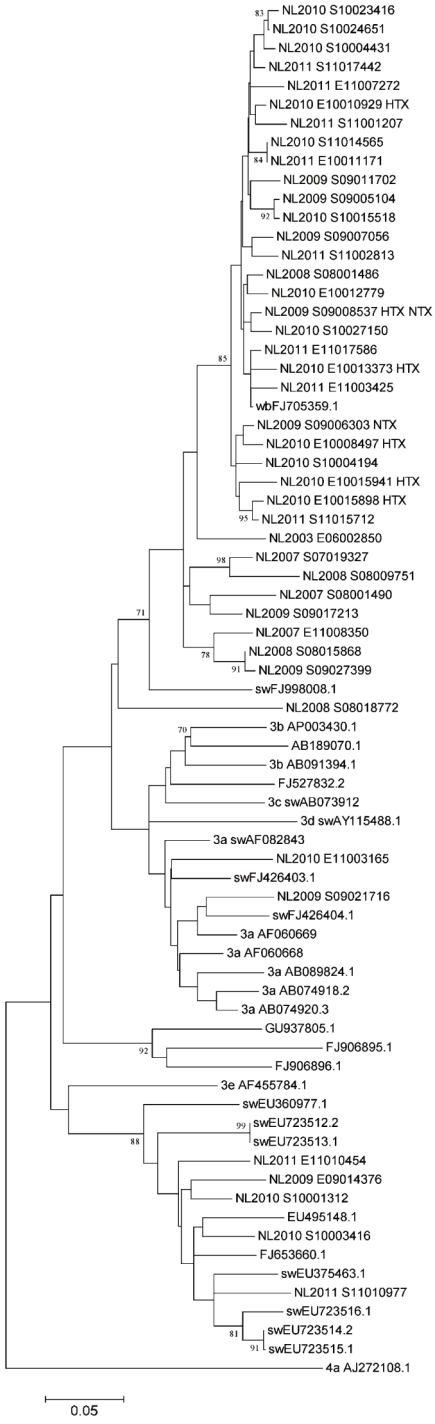

Figure 2 Phylogenetic tree of ORF1 HEV sequences in six chronic HEV infected heart transplant recipients. Phylogenetic relation of 306 bp ORF1 region was calculated using Maximum likelihood, K2P analysis with bootstrapping (n=1000). Branch lengths are proportional to the evolutionary relationship between the sequences and internodal confidence of >70% is depicted in the tree. Heart transplant recipients and heart-kidney transplant recipients are indicated in the taxa with respectively HTX and HTX KTX. HEV sequences of Dutch origin90 and year of infection are indicated as NLyyyy-isolate number, Genbank accession numbers are no JQ15401, JQ15406-JQ15417, JQ15419-JQ15422, JQ15426 and JQ15429-JQ15448. No indication for a common origin, nor for nosocomial HEV transmission was found.

Liver histology

All chronic HEV infected patients underwent a liver biopsy. Typical signs of acute viral hepatitis were seen with inflammatory activity, councilman bodies and acidophilic degeneration (Figure 3). Inflammatory activity was predominantly located in the periportal area (Figure 3A). Histology Activity Index scores [131] were calculated for all biopsies (Table 2). Advanced fibrosis was seen in four patients (67%) of whom three had been infected with HEV for less than one year. The lowest HAI score (sum=1) belonged to the patient in whom a liver biopsy was taken only 5 months after the first HEV RNA positive sample in this patient. All biopsies displayed some steatosis, but in four of the six biopsies a marked steatosis ranging from 5 to 25% was seen.

Table 2 Liver biopsy results in six chronic HEV infected HTX recipients

	*Infection time in months**	*HAI**-Knodell score*				*Total HAI score*	*Steato-sis†*	*Cholestasis*	Iron‡
		Peri-portal necrosis	Intra-lobulair inflam-mation	Portal inflam-mation	Fibrosis				
1	9	1	3	2	3	9	0	none	1+
2	5	1	2	1	3	7	1	focal	none
3	7	3	3	3	3	12	0	none	none
4	5	0	1	0	0	1	1	none	none
5	22	3	3	3	3	12	1	none	none
6	8	1	1	1	1	4	1	none	none

*Calculated from time point at which first sample was positive for HEV RNA up to biopsy date
**HAI=Histology Activity Index 131, †According to Brunt et al.132, ‡According to Brunt et al.133

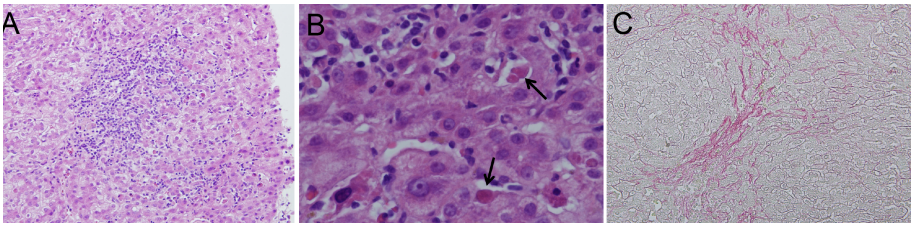

Figure 3 Histological presentation of chronic hepatitis E infection in heart transplant recipients. (A) Liver biopsy showing a typical pronounced portal lymphocytic infiltration with several plasma cells. Some minor lobular infiltration is present. Degeneration of hepatocytes can be seen with the typical presence of councilman bodies. (B) Detail of 3A: The arrows indicate an acidophilic hepatocyte degeneration with typical presence of Councilman bodies. (C): Pronounced portal fibrosis with septa.

Clinical course and management

An overview of the clinical course and management of the chronic HEV infected patients including serum through levels of tacrolimus, dose reduction of concomitant immunosuppressant therapy and treatment with orally administrated ribavirin is given in Figure 4. Liver enzyme values were elevated in all cases, with a median peak ALT level of 356 U/l (range 81-817), AST level of 230 U/l (range 66-672), Alkaline phosphatase level of 170 U/l (range 80-278) and γ-GT level of 308 U/l (range 196-1740). bilirubin levels were elevated in three patients [17, 18 and 95 μmol/l). All patients that had peak ALT levels of more than four times the upper limit of normal (ULN male=40 U/l; female 30 U/l) had advanced liver fibrosis on presentation. One patient (see Figure 4D) experienced only moderate elevation of liver enzymes throughout the whole course of HEV infection with peak ALT (81 U/l) of two times the upper limit of normal. Interestingly, patient 2 had a peak ALT level of more than 20 times the ULN (817 U/l), but was the only patient to clear the chronic HEV infection spontaneously (Figure 4B). No dose reduction of immunosuppressive therapy was possible or necessary in this patient. Moreover, patient 2 was the only patient that had IgG seroconversion before IgM seroconversion. IgG seroconversion occurred 127 days before IgM seroconversion and was accompanied with subsequent rapid increase of HEV viral load. At the time of IgM seroconversion HEV RNA had almost become undetectable.

Immunosuppressive therapy was reduced in the other patients. Patient 3 managed to clear the HEV infection after dose reduction of tacrolimus (Figure 4C). A decrease in HEV viral load was seen after mycophenolate mofetil (MMF) was stopped in patient 1 (Figure 4A) and after a subsequent three-month course of low-dose ribavirin therapy, patient 1 was able to rapidly clear the HEV infection. An almost similar course could be observed in patient 4 and 6 (Figure 4D and F respectively). In these two patients tacrolimus dose was slightly reduced, but both cleared the virus only after a stepwise dose increase of ribavirin and were therefore treated for 9 and 8 months respectively. Interestingly, patient 6 became IgM antibody negative immediately after clearing the HEV infection, but remained IgG antibody positive during follow up. Patient 5 (Figure 4E) did have a drop in ALT after dose reduction of tacrolimus, but no effect was seen with respect to the HEV viral load. After introduction of ribavirin, the viral load initially dropped, but increased for unknown reasons after 3 months. There were no indications that this patient was non-compliant to the ribavirin therapy. At the time of writing, patient 6 was treated for 9 months with ribavirin including several increases in dosage, though HEV RNA was still detectable in last follow-up sample.

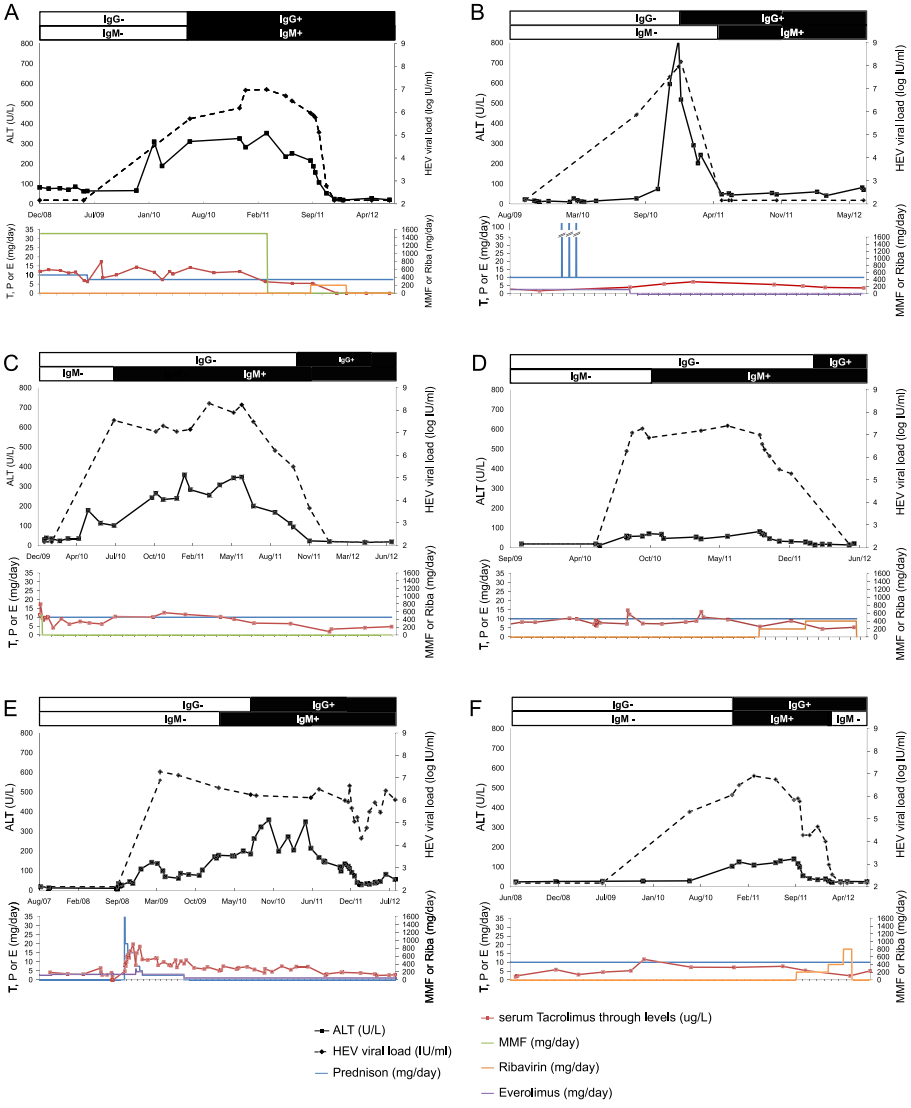

Figure 4 Clinical course of chronic HEV infection in six heart transplant recipients. (A) Patient 1. (B) Patient 2: received three times R-CHOP for a lymphoma, explaining the peaks in prednisolone. (C) Patient 3. (D) Patient 4 . (E) Patient 5: received a kidney transplant in 2008, explaining the increase in immunosuppressant therapy. (F) Patient 6. The ALT upper limit of normal is 30U/L and 40U/L for females and males respectively. The HEV RNA lower limit of detection is 143 (=2.16 log) IU/ml.

DISCUSSION

We studied six chronic HEV infected heart transplant recipients in depth to gain more insight in clinical, biochemical and virological presentation. The six patients represented 2.3% of 263 HTX recipients of whom HEV infection was identified and confirmed by real-time RT-PCR and serology. All patients developed chronic HEV infection and phylogenetic analysis classified all isolates within genotype 3. No direct relations between the isolated viruses were shown, thereby excluding a common source in our patients.

Production of HEV specific IgM and IgG antibodies was delayed up to 301 and 539 days respectively after HEV RNA was detectable for the first time. This finding supports previous reports that testing for HEV infection in transplant recipients with elevated liver enzymes should be performed by HEV RNA (real time RT-PCR) rather than screening for antibodies [112, 123].

Sixty-seven percent of the chronic infected patients had already progressed to advanced fibrosis within two years after initial infection. Rapid progression of fibrosis in HEV infected transplant recipients has been described before [112, 113, 122]. We cannot exclude the presence of some pre-existing liver disease e.g. due to right sided heart failure before transplantation or as a consequence of drug induced liver disease post-transplantation. However, 4 out of 6 patients had normal liver enzyme values before the first positive HEV PCR. The current study again emphasizes that early detection, and where possible intervention, is needed to prevent severe liver damage.

All (chronic) HEV infected patients had elevated liver enzyme values shortly after HEV RNA was first detectable in serum. Therefore, in patients on immunosuppressive drugs, hepatitis E virus infection should always be part of the differential diagnosis of all raised liver enzymes. Interestingly, a high peak ALT level (>20 times the ULN) was seen in the only patient that spontaneously cleared the chronic HEV infection. Previously, in a group of 85 HEV infected transplant recipients, clearance of HEV infection within 6 months was associated with higher peak ALT levels compared to transplant recipients that did not clear the HEV infection within 6 months [71]. Our study showed that even after an infection duration of more than 6 months, HEV can be cleared spontaneously in immunocompromised patients.

Previous studies have indicated that non-travel-associated HEV infection route is thought to be zoonotic of origin[15, 105]. Indeed, we found that the non-immunocompromised spouses of the HEV infected patients in this study did not have an active infection or experienced seroconversion, in accordance with the theory that person-to-person infection is unlikely to occur.

In our cohort, all patients received tacrolimus based immunosuppressive therapy. Previously, tacrolimus has been described as a risk factor for developing chronic HEV infection in solid organ transplant recipients [71]. The first step in the approach of chronic HEV infected patients is, where possible, a reduction of immunosuppressive therapy. In this study, dose reduction led to clearance of the HEV infection in one patient and in a decline in the viral load of HEV in other patients. However, in the majority of patients, complete clearance of HEV infection only followed treatment with ribavirin. An approach with stepwise dosing of ribavirin seems therefore reasonable to minimize side effects and optimize effectively. Low-dose ribavirin was able to normalize liver enzymes within weeks, but in all patients viral clearance took longer. The time correlation between ribavirin therapy and reduction in viral load and liver parameters adds further weight to the effectiveness of ribavirin in HEV infection in this patient group.

A clear limitation, due to design, of our study is, that earlier cleared infections may have been missed.

In conclusion chronic HEV infection in heart transplant recipients may lead to rapid fibrosis of the liver. The current study highlights the need for early detection of HEV infection in immunocompromised patients and the importance of early medical intervention if possible by reducing immunosuppressive therapy and if insufficient, by introduction of ribavirin. We advise additional HEV RNA screening in immunocompromised patients with elevated liver enzymes, since antibody production is often delayed.

ACKNOWLEDGMENTS

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Chapter 3.3

Hepatitis E virus: an underestimated opportunistic pathogen in recipients of allogeneic hematopoietic stem cell transplantation

Suzan D. Pas*, Jurjen Versluis*, Hendrik J. Agteresch, Robert A. de Man, Jolanda Maaskant, Marguerite E.I. Schipper, Albert D.M.E. Osterhaus, Jan J. Cornelissen, and Annemiek A. van der Eijk

*Both authors contributed equally

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ABSTRACT

Hepatitis E virus (HEV) is increasingly acknowledged as a cause of hepatitis in healthy individuals as well as immunocompromised patients. Little is known of HEV infection in recipients of allogeneic hematopoietic stem cell transplantation (alloHSCT). Therefore, we set out to study the incidence and sequelae of HEV as a cause of hepatitis in a recent cohort of 328 alloHSCT recipients. HEV-RNA was tested in episodes of liver enzyme abnormalities. In addition, HEV-RNA and HEV serology were assessed pre- and post-alloHSCT. We found 8 (2.4%) cases of HEV infection, of which 5 had developed chronic HEV infection. Seroprevalence pre-alloHSCT was 13%. 4 patients died with HEV viremia, with signs of ongoing hepatitis, having a median time of infection of 4.1 months. The 4 surviving patients cleared HEV after a median period of 6.3 months. One patient was diagnosed with HEV reactivation after a preceding infection prior to alloHSCT. While the incidence of developing acute HEV post-alloHSCT is relatively low, the probability of developing chronic hepatitis in severely immunocompromised patients is high. Therefore, alloHSCT recipients should be screened pre-transplantation by HEV serology and RNA. Furthermore, a differential diagnosis including hepatitis E is mandatory in all alloHSCT patients with severe liver enzyme abnormalities.

INTRODUCTION

In 1983, a new waterborne hepatitis agent was found after an outbreak of unexplained hepatitis at a military camp, later identified as Hepatitis E Virus (HEV). HEV is endemic in resource-limited countries and an emerging health issue in industrialized countries.[16, 105] It is a causative agent of acute and chronic hepatitis, transmitted via fecal-oral route, with a mostly self-limiting course in healthy individuals. In human HEV infection, there are four known genotypes prevalent, with genotypes 1 and 2 responsible for large waterborne HEV outbreaks in developing countries (Africa and Asia), and genotypes 3 and 4 generally seen in sporadic cases as a zoönotic infection in industrialized countries.[16, 134] Since the first evidence of chronic hepatitis due to HEV in recipients of solid organ transplants, an increasing awareness for HEV has become apparent.[69, 112]
Persistent chronic infection and cirrhosis have been reported in immunocompromised patients, with most cases in solid organ transplant recipients.[69] However, HEV was recently also reported in recipients of allogeneic stem cell transplantation (alloHSCT).[135-138] A prevalence of 1-3% of hepatitis E viremia in recipients of solid organ transplants has been reported, with 47-83% of the patients developing chronic hepatitis.[71, 89, 90, 139] So far, the incidence and sequelae of hepatitis due to HEV in recipients of alloHSCT is largely unknown.
Following two recent cases of HEV infection in our clinic, we set out to retrospectively evaluate the point prevalence and clinical sequelae of HEV infection in a cohort of alloHSCT recipients in our clinic, and we studied the role of HEV in transplant recipients presenting with liver enzyme abnormalities.

MATERIALS AND METHODS

Sample collection

We conducted a retrospective cross-sectional analysis of all adult alloHSCT recipients transplanted in the period January 2006 to July 2011, whose serum or EDTA-plasma samples were available in the biobank of Erasmus MC, Rotterdam, The Netherlands. These samples, stored at -20°C or -80°C, had been collected during routine visits to our outpatient clinic for clinical assessment of cytomegalovirus (CMV) and Epstein-Barr virus (EBV) reactivation. To select samples, a Laboratory Information Management System database search was performed for last pre-transplantation and most recent post-transplantation sample availability. In addition to the cross-sectional analysis, samples were

selected from patients experiencing episodes with alanine transaminase (ALT) abnormalities grade 2 to 4, according to Common Terminology Criteria for Adverse Events v3.0 (CTCAE). CTCAE grade 2 to 4 ALT abnormalities are defined as at least 2.5 times the upper limit of normal. This study was approved by the medical ethical committee of Erasmus MC, Rotterdam, The Netherlands (MEC approval: 2012-522).

Virological parameters

For detection of both HEV specific IgM and IgG in serum or plasma samples the commercially available HEV ELISAs (Wantai, Beijing, PR China) were used. Available peripheral blood, feces and cerebrospinal fluid (CSF) samples of HEV-RNA positive patients were retrospectively analyzed during the course of infection to study the kinetics of serum antibody responses (IgM and IgG) and viremia in different body compartments.

All samples were screened for HEV-RNA by an internally controlled quantitative real-time RT-PCR, described previously.[13] The RT-PCR had a lower limit of detection (95% hit rate) of 143 (2.16 log) IU/ml as determined by the 1st WHO standard for HEV RNA NAT-Based assays (6329/10, Paul Ehrlich Institute, Germany). Phylogenetic analysis was performed to determine genotype, to exclude a common source of infection, and to examine potential HEV reactivation. Statistical analysis and data collection were performed using Microsoft Office Excel 2007 and SPSS v20.

Case definition

A case of HEV infection was defined as a patient with a HEV RNA positive serum or plasma sample, and was confirmed either by showing HEV specific serum IgM or IgG antibody response, or by showing the presence of HEV RNA in sequential samples. Chronic infection was diagnosed by retrospective testing of stored samples of identified cases and was defined as having HEV viremia of more than six months.

RESULTS

Patient characteristics

A total of 207 episodes of acute ALT abnormalities, occurring in 138 out of 328 alloHSCT recipients, were evaluated, in addition to a cross-sectional RT-PCR analysis of all 328 patients (Figure 1). As delineated in Table 1, the cohort included 178 (54%) male and 150 (46%) female patients with a median age at transplantation of 50 (range: 17-66) years. Stem cell sources included sibling donors (n=145, 44%), adult matched unrelated donors (MUD) (n=137, 42%) and Umbilical Cord Blood (UCB) grafts (n=46, 14%). Acute myeloid leukemia was the most frequent diagnosis for transplantation (n=142, 43%), followed by acute lymphoblastic leukemia (n=49, 15%), and non-Hodgkin's lymphoma (n=31, 9%). All patients received graft versus host disease (GVHD) prophylaxis with a combination of a calcineurin inhibitor (cyclosporine A) and mycophenolate according to local policy. Acute GVHD grade II-IV occurred in 130 (40%) patients, and chronic extensive GVHD was present in 122 (37%) patients. At the time of analysis (2012, December), 180 (55%) patients were still alive, with a median follow-up of 40.9 (range: 10-77) months from alloHSCT.

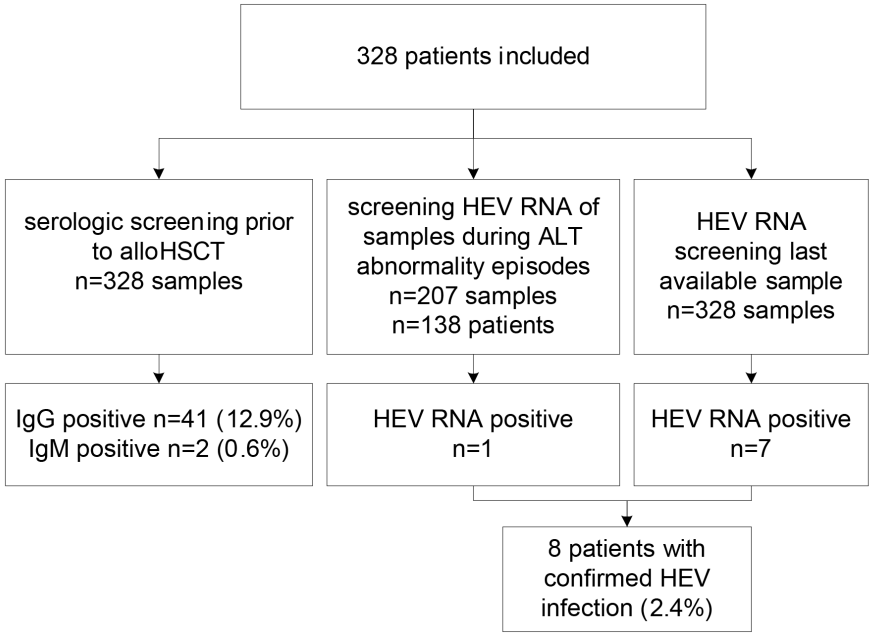

Figure 1 Overview of sample selection and study results

Table 1 Patient characteristics of the cohort (n=328)

Characteristic	Number
Age of Transplantation (years)	
Median (range)	50.4 (17-66)
Sex, number (%)	
Male	178 (54%)
Female	150 (46%)
Diagnosis, number (%)	
AML	142 (43%)
ALL	49 (15%)
NHL	31 (9%)
CLL	24 (7%)
MM	18 (5%)
MDS	16 (5%)
Other	48 (15%)
Type of allogenic transplantation, number (%)	
UCB	46 (14%)
MUD	137 (42%)
SIB	145 (44%)
GVHD, number (%)	
Acute grade I	42 (13%)
Acute grade II - IV	130 (40%)
Chronic limited	32 (10%)
Chronic extensive	122 (37%)
Patients alive	
Number (%)	180 (55%)
Time to follow-up (months)	
Median (range)	40.9 (10-77)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; GVHD, graft versus host disease; MDS, myelodysplastic syndrome; MM, multiple myeloma; MUD, matched unrelated donor; NHL, non-Hodgkin's lymphoma; SIB, sibling; UCB, umbilical cord blood.

Virological parameters

In total, eight (2.4%) cases of confirmed HEV infection were found in 328 patients, of which seven (88%) were identified by cross-sectional analysis, and one (13%) by screening the episodes of acute ALT abnormalities.

HEV specific IgG prior to alloHSCT was detected in 41 (13%) patients. Two (0.6%) patients were IgM positive, though HEV viremia could not be confirmed by RT-PCR. Presence or absence of HEV specific antibodies (both IgM and IgG) prior to alloHSCT was not predictive for HEV infection after alloHSCT, tested by Pearson's chi square- test of independency (p=0.313).

The courses of HEV infection of all eight cases are presented in Figure 2. Clinical and virological features are delineated in Table 2. Patients will be annotated according to their assigned letter: 'patients A–H'. Within the eight cases, complete HEV IgM and IgG seroconversion occurred in five patients, of whom four eventually cleared the virus and one deceased with a HEV viremia (patient A–C,F,H). Median time from first HEV RNA detection to HEV-IgM and HEV-IgG conversion of these patients was 65 (range: 0-245) days, and 126 (range: -594-351) days, respectively. Three patients, who all died with HEV viremia, had aberrant serodynamics: one patient did not have detectable HEV-IgG, with only one serum sample testing HEV-IgM positive (patient G). Two patients did not have detectable HEV-IgM levels (patients D,E). One of them had detectable HEV-IgG in only one sample (patient D) and one had detectable HEV-IgG levels at time of alloHSCT, though declining to undetectable at the time of death seven months later (patient E).

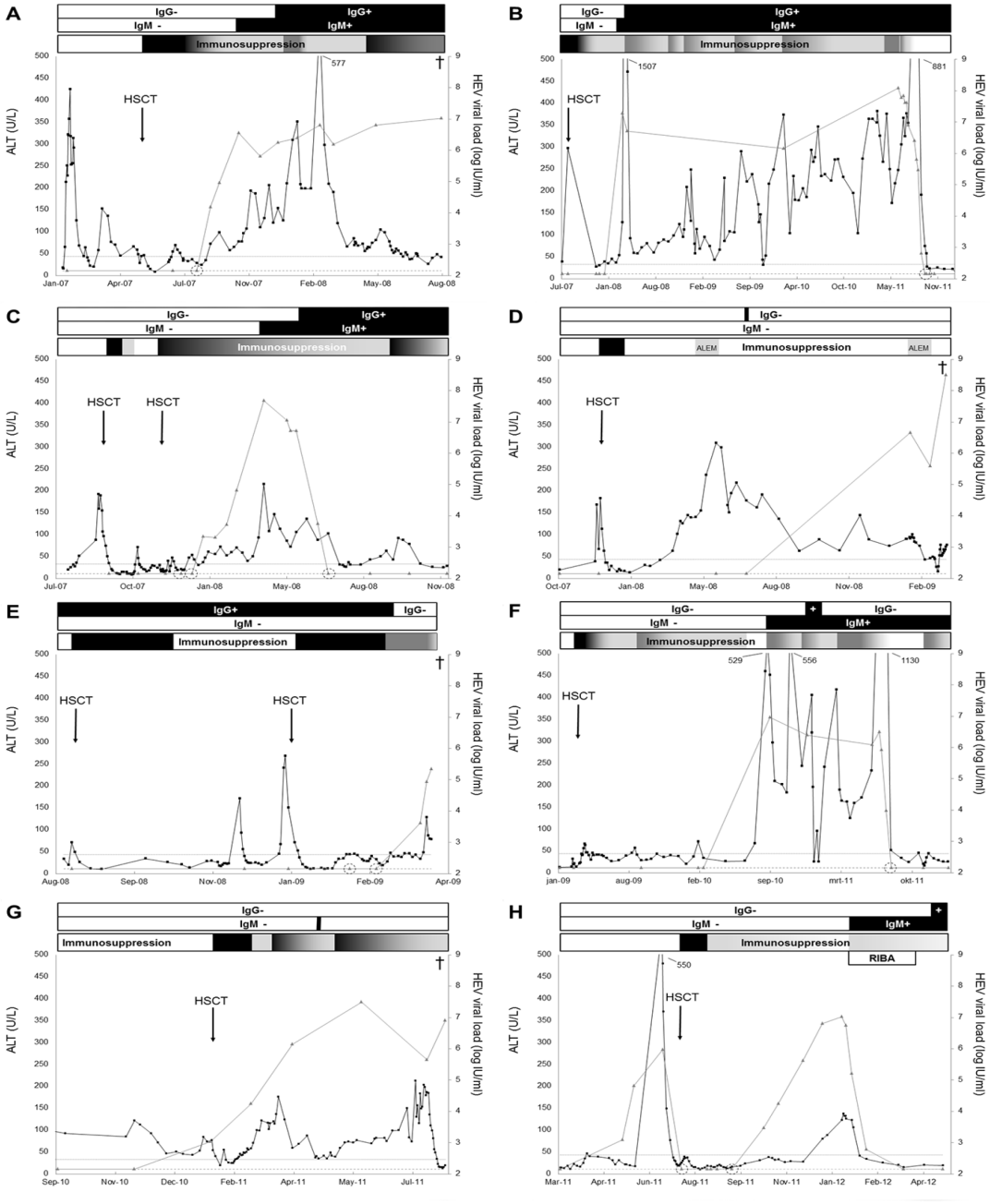
A
IgG-
IgG+
IgM -
IgM+
Immunosuppression
HSCT
577
†
ALT (U/L)
HEV viral load (log IU/ml)
Jan-07
Apr-07
Jul-07
Nov-07
Feb-08
May-08
Aug-08
B
IgG-
IgG+
IgM -
IgM+
Immunosuppression
HSCT
1507
881
ALT (U/L)
HEV viral load (log IU/ml)
Jul-07
Jan-08
Aug-08
Feb-09
Sep-09
Apr-10
Oct-10
May-11
Nov-11
C
IgG-
IgG+
IgM -
IgM+
Immunosuppression
HSCT
HSCT
ALT (U/L)
HEV viral load (log IU/ml)
Jul-07
Oct-07
Jan-08
May-08
Aug-08
Nov-08
D
IgG-
IgM -
ALEM
Immunosuppression
ALEM
HSCT
†
ALT (U/L)
HEV viral load (log IU/ml)
Oct-07
Jan-08
May-08
Aug-08
Nov-08
Feb-09
E
IgG+
IgG-
IgM -
Immunosuppression
HSCT
HSCT
†
ALT (U/L)
HEV viral load (log IU/ml)
Aug-08
Sep-08
Nov-08
Jan-09
Feb-09
Apr-09
F
IgG-
+
IgG-
IgM -
IgM+
Immunosuppression
529
556
1130
HSCT
ALT (U/L)
HEV viral load (log IU/ml)
jan-09
aug-09
feb-10
sep-10
mrt-11
okt-11
G
IgG-
IgM -
Immunosuppression
HSCT
†
ALT (U/L)
HEV viral load (log IU/ml)
Sep-10
Nov-10
Dec-10
Feb-11
Apr-11
May-11
Jul-11
H
IgG-
+
IgM -
IgM+
Immunosuppression
RIBA
550
HSCT
ALT (U/L)
HEV viral load (log IU/ml)
Mar-11
Apr-11
Jun-11
Aug-11
Sep-11
Nov-11
Jan-12
Feb-12
Apr-12
ALT (U/L)
ALT ULN (U/L)
HEV viral load (log IU/ml)
LOD HEV PCR (2.16 log IU/ml)
HEV viral load <2.16 log IU/ml, pos
Intensive immunosuppression
Intermediate immunosuppression
Mild immunosuppression
No immunosuppression

Figure 2 Courses of hepatitis E infection in all eight individual patients. (A) HEV RNA persisted, although HEV-IgM and HEV-IgG seroconversion occurred under immunosuppressive therapy. This patient deceased of therapy refractory progressive gastro-intestinal GVHD with concurrent chronic HEV infection. (B) Acute ALT abnormalities arose during HEV infection. This patient was mistakenly diagnosed as hepatic GVHD, and immunosuppression was intensified multiple times because of persisting liver enzyme abnormalities. Patient cleared HEV with stopping all immunosuppression, after the diagnosis of HEV infection in this study. (C) This patient developed primary graft failure of a 8/8 HLA-matched unrelated donor graft after reduced intensity condition with rabbit antithymocyte globulin, fludarabine, and a single donor fraction of 2 gray total body irradiation. HEV RNA was present after second alloHSCT. This patient cleared the infection after HEV-IgM and HEV-IgG seroconversion, supported by reduction of immunosuppressive therapy.(D) This patient developed graft failure of a 7/8 HLA-matched unrelated donor graft after reduced intensity conditioning with rabbit antithymocyte globulin, fludarabine, and a single fraction of 2 gray total body irradiation. Patient's disease relapsed three months after graft failure. Reinduction therapy was started with alemtuzumab (ALEM) and a second alloHSCT was prepared. However, due to recurrent infections, patient was not able to complete treatment. Patient died shortly after his second cycle of alemtuzumab because of complications of a meningitis and secondary sepsis with Escherichia coli. Of note, patient's CSF samples tested positive for HEV.

Table 2 Patient characteristics of hepatitis E–confirmed patients (n=8)

Patient	Sexe	Age at allo-HSCT (yrs)	Diag-nosis	Stem cell source	Alive at EOF	GVHD	Initial diag-nosis	At time of alloHSCT		HEV gt	Time from alloHSCT to infection (mos)	Duration of infection (mos)	Median (range) ALT levels during infection (U/l)†	Immune-suppression at time of infection	Hepatic fibrosis in liver histology	Blood products received <3 mos to infection
								HEV RNA	IgG status							
1	M	44	AML	UCB	no	Acute grade II-IV Chronic extensive	GVHD	-	-	3	2.7	12.5*	73 (24-577)	ciclosporin, prednisone, mycophenolate	N/A	21
2	F	54	NHL	MUD	yes	Acute grade II-IV Chronic limited	GVHD	-	-	3	8.4	42.4	207 (31-1507)	ciclosporin	F3	0
3	F	59	MDS	MUD	yes	Acute grade II-IV Chronic extensive	GVHD	-	-	3	3.4	6.3	72 (36-215)	ciclosporin, mycophenolate	N/A	48
4	M	43	CLL	MUD	no	None	DILI	-	+	3	14.0	1.6*	66 (15-309)	alemtuzumab	N/A	3
5	M	66	AML	MUD	no	Acute grade II-IV	DILI	-	+	3	5.8	1.7*	39 (19-268)	ciclosporin, prednisone, mycophenolate	N/A	35
6	M	58	NHL	MUD	yes	Acute grade II-IV Chronic extensive	GVHD	-	+	3	18.3	11.3	208 (25-1130)	sirolimus, prednisone	F1	0
7	F	39	SAA	UCB	no	Acute grade II-IV	DILI	+	-	3	0	6.5*	70 (12-213)	ciclosporin, mycophenol acid	F0	21
8	M	59	AML	UCB	yes	None	GVHD	+	-	3	-2.0	2.1 and 4.9	27 (10-550)	none	N/A	50

Abbreviations: alloHSCT, allogeneic hematopoietic stem cell transplantation; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; DILI, drug induced liver injury; EOF, end of follow-up; GVHD, graft-versus-host disease; HEV gt, hepatitis E virus genotype; MDS, myelodysplastic syndrome; MUD, matched unrelated donor; N/A, not available; NHL, non-Hodgkin's lymphoma; SAA, severe aplastic anemia; SIB, sibling; UCB, cord-blood.* Patient died having a HEV viremia† ALT Upper limited of normal, male = 44 U/l or female = 33 U/l

HEV-Open Reading Frame 1b (ORF1b) sequences were generated of all eight cases and deposited in Genbank under the accession numbers JQ015439, JQ015407, KC171439-KC1714444, KC171447, KC171450 and KC171451. Phylogenetic analysis did not identify a common or nosocomial source of HEV transmission. All HEV isolates were classified within genotype 3, as shown in the phylogenetic tree (Figure 3). Interestingly, confirmed HEV reactivation occurred in one patient, as described below (patient H).

Characteristics of HEV RNA positive patients

The median age of eight HEV infected patients was 56 (range 39-66) years at transplantation, including five (63%) males and three (37%) females (Table 2). All patients were screened for hepatitis B virus, hepatitis C virus, EBV, adenovirus, varicella zoster virus, herpes simplex virus type 1 and 2, and CMV by PCR to exclude the role of other potential hepatrophic viruses. All tested samples were undetectable by PCR, except for one patient experiencing CMV reactivation at the time of HEV infection (patient E). In this patient, HEV viremia persisted after successful treatment with ganciclovir, excluding the role of CMV in hepatitis in this patient. All eight patients received a graft from an alternative donor, including peripheral blood grafts from an adult MUD in five patients (63%) and UCB grafts in three (37%) patients. Plasma's of the adult MUD grafts were HEV RNA negative. No samples of the UCB grafts were available for HEV RNA screening, yet two of three UCB recipients were HEV viremic at the time of alloHSCT (patient G,H). Six patients received multiple blood transfusions within three months prior to HEV infection, including platelet and red blood cell transfusions. None of the blood products were available for testing for HEV serology or RNA at the time of submission.

The median time from alloHSCT to infection was 4.6 (range: -2-18) months. The median peak ALT during HEV infection was 289 (range: 138-1507) U/l. At the time of infection, six (75%) patients were receiving intensive immunosuppressive therapy (≥2 agents), prescribed for GVHD prevention (n=2, 33%) or GVHD treatment (n=4, 66%). In the HEV infected patients, liver enzyme abnormalities were thought to be related to hepatic GVHD in five (63%) patients, and drug induced liver injury in three (38%) patients.

Four (50%) patients died with persistent HEV viremia and signs of ongoing hepatitis (patient A,D,E,G). Median duration of HEV infection in deceased patients was 4.1 (range: 2-13) months, with acute HEV infection in three patients and chronic HEV infection in one patient. The cause of death was respiratory failure due to infection (fungal, bacterial and viral) in three patients (patient

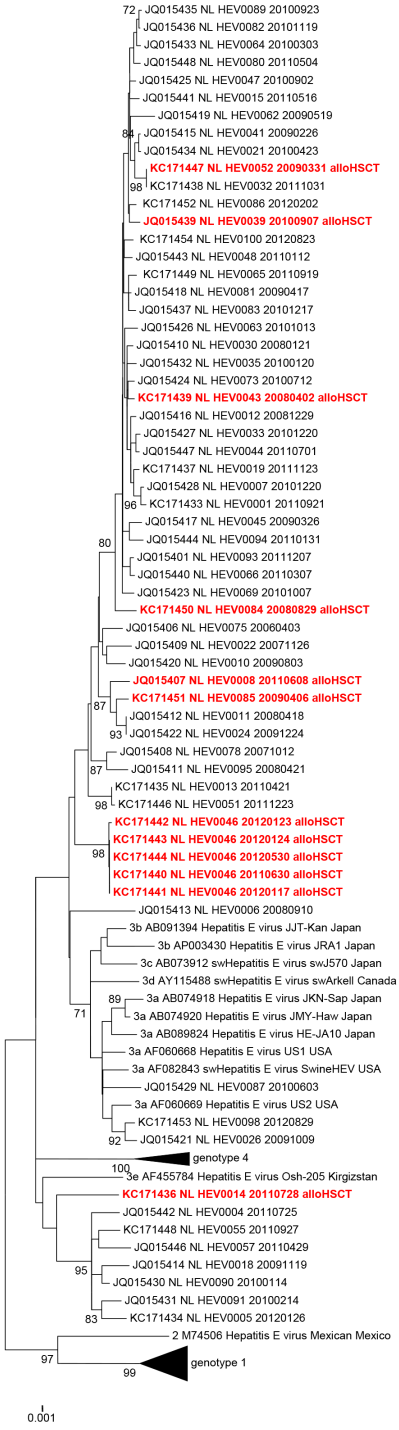

Figure 3 Phylogenetic tree of ORF1b HEV sequences **in eight HEV infected alloHSCT recipients.** Phylogenetic relation of 321 bp ORF1b region was calculated using Maximum likelihood, K2P analysis with bootstrapping (n=1000). Branch lengths are proportional to the evolutionary relationship between the sequences and internodal confidence of >70% is depicted in the tree. Genbank accession numbers, country of origin (e.g. NL), HEV study number (e.g. HEV001) and date of drawal (yyyymmdd) and AlloHSCT recipients are indicated in the taxa (red text). No indication for a common origin or for nosocomial HEV transmission was found.

D,E,G), and one patient died of therapy refractory progressive gastro-intestinal GVHD (patient A). Of note, one of the deceased patients appeared to have HEV RNA positive cerebrospinal fluid (CSF) with retrospective testing of CSF samples (patient D). These samples were obtained during an episode of meningitis and secondary sepsis with positive CSF and blood cultures for *Escherichia coli*. Radiological evaluation (CT-scan) revealed cerebral ischemia due to infection. This patient eventually died of respiratory failure due to fluid aspiration with a low level of consciousness since the meningitis.

The four (50%) living patients cleared HEV infection within a median period of 6.3 (range: 2-42) months (patient B,C,F,H). One patient received ribavirin treatment twice daily with 400 milligram for three months after a starting dose of three times 600 milligram daily for ten days because of a concurrent respiratory syncytial virus infection (patient H). Three patients cleared HEV during cessation of immunosuppressive therapy (patient B,F,H). The cessation rate depended on the presence and/or occurrence of GVHD. Among living patients, chronic HEV occurred in three patients (patient B,C,F), whereas one patient was able to clear HEV infection within six months (patient H). After HEV diagnosis was confirmed, a liver biopsy was taken from two patients (patient B,F), showing hepatitis, severe fibrosis, and portal inflammation (Figure 4). Liver histology was available in one patient by autopsy, showing no abnormalities (patient G).

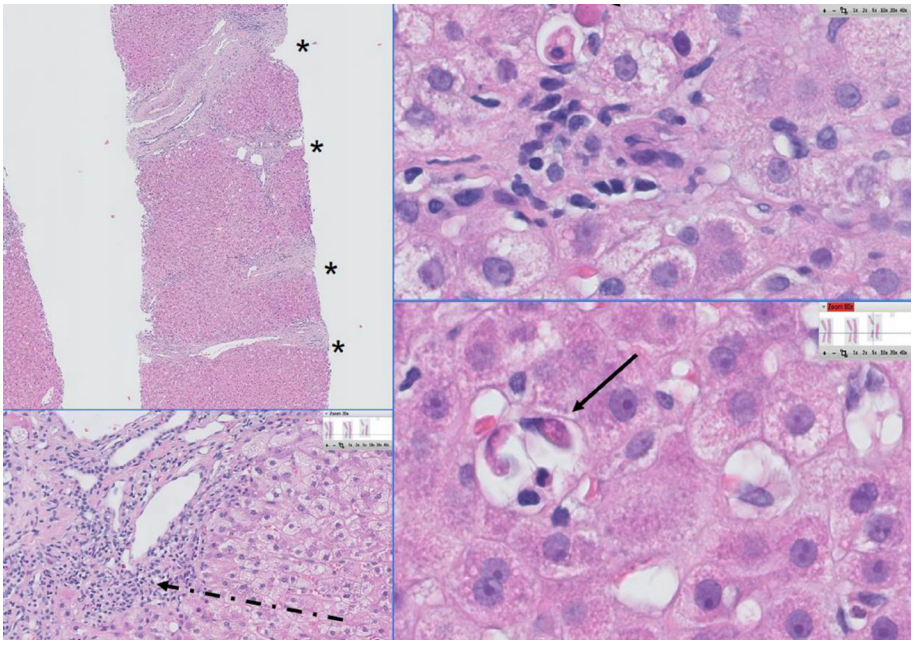

Figure 4 Liver histology of a patient with chronic HEV. The histopathology of chronic **HEV** infection in this patient is characterized by a dense lympho-plasmocellular infiltrate (dashed arrow) in the portal tracts, combined with severe fibrosis (F3) and porto-portal septation (*). Multiple foci of apoptotic bodies are seen in the lobuli surrounded by a few inflammatory cells, indicating individual hepatocyte necrosis (Councilman-bodies: arrow) and probably caused by viral replication.

Remarkably, one patient initially cleared the virus and showed reactivation after a period of 53 days of undetectable HEV RNA (patient H). At the time of alloHSCT, HEV RNA was detectable, though viral load was low (<143 IU/ml). The second viremic period was characterized as viral reactivation after alloHSCT, based on identical HEV-ORF1b sequences (Figure 3). This patient finally cleared the reactivated HEV infection within two months after diagnosis, supported by ribavirin treatment (as described above) and reduction of immunosuppressive therapy.

DISCUSSION

Recipients of allogeneic stem cell grafts, and especially those receiving alternative donor grafts are at increased risk of opportunistic bacterial, fungal and viral infections. Here we describe the first retrospective cross-sectional study of hepatitis E virus infection in a large cohort of alloHSCT patients. We report a relatively low incidence of 2.4%, as compared to other opportunistic infections in alloHSCT recipients. Nevertheless, we found a high probability of 63% of developing chronic HEV infection.

Previously, two cohorts of 72 and 52 alloHSCT patients were screened for HEV by Abravanel *et al*.[135] and Koenecke *et al*.[137] respectively, without positive cases for HEV infection or reactivation, concluding that alloHSCT patients are at low risk for HEV infection and reactivation. However, these two cohorts of alloHSCT recipients comprised a more limited number of patients. In our study we identified eight HEV cases in a larger cohort (n=328), confirming the HEV prevalence of 2.4% in immunocompromised patients.[69, 89, 90, 112] Secondly, the study of Abravanel *et al*.[135] included a restricted follow-up period of six months after alloHSCT, while our study had a median follow-up time of 41 months. Additionally, misdiagnosing HEV as drug induced liver injury has been reported previously by Dalton *et al*.[121], whereas this patient group was excluded in the study of Koenecke *et al*.[137] To reduce the risk of missing HEV infections, we screened all patients for HEV RNA at episodes of liver enzyme abnormalities in addition to last available samples. Of the confirmed HEV cases, five were misdiagnosed as GVHD, and three cases were mistakenly diagnosed as drug induced liver injury. Diagnosis of HEV in these patients is hampered by relatively low peak aminotransferase levels compared to non-immunocompromised patients,[68] which may be explained by intensive immunosuppressive therapy suppressing inflammation.

In our cohort, chronic hepatitis occurred in five out of eight acute HEV cases. However, only six patients had sufficient follow-up for a potential diagnosis of chronic hepatitis, because two patients died within two months after acquiring HEV infection. Progression to chronic HEV in alloHSCT patients may be explained by an impaired immune reconstitution, including insufficient lymphocyte recovery, which are well known risk factors for post-transplantation infections.[140-142] In particular impaired reconstitution of $CD4^+$ and $CD8^+$ T-cells predispose for infectious morbidity,[143] which is confirmed in studies with CMV and EBV viremia, with patients having low specific CMV and EBV $CD4^+$ and $CD8^+$ T-cell counts predisposing for CMV and EBV reactivation, respectively.[144, 145]

Phylogenetic analysis of patient derived HEV sequences before and after alloHSCT established HEV reactivation in one patient. This is the second case of HEV reactivation after alloHSCT described so far in literature.[136] We could not unequivocally demonstrate a reinfection or reactivation in three viremic patients having detectable IgG prior to alloHSCT, since no HEV RNA was detected in available samples prior to alloHSCT. Four other patients were seronegative prior to transplantation, suggesting that transmission had occurred after alloHSCT.

In industrialized countries, HEV genotype 3 predominantly infects pigs, wild boars and deer but also humans, and is recognized as a zoönotic agent. However the main modes of transmission of genotype 3 and 4 viruses remain to be determined.[105, 134] The source of HEV infection is unclear, but HEV transmission may be enterically (food borne: porcine livers, shellfish), via blood or blood products, mother-to-child, and although rare human-to-human.[16, 52] Donors and donated blood are not routinely tested for HEV RNA worldwide, although reports of several cohorts in different countries of healthy blood donors reported HEV RNA and HEV IgM reactivity, suggesting active infection. [65, 146, 147]

In our cohort, transmission of HEV by blood products cannot be excluded because six out of eight viremic patients received multiple blood transfusions. Unfortunately none of these blood products were available for testing at the time of submission. The high probability of developing chronic HEV found in this study was consistent with other studies in recipients of solid organ transplants.[69, 89, 90, 112] HEV infected patients are at risk (67%) of progression to fibrosis in one year from infection,[89] and also cirrhosis (10%).[71] Therefore immunocompromised patients should be screened prior to transplantation, and during episodes of liver enzyme abnormalities post-transplantation. In our study patients showed aberrant serology, which may be explained due to their impaired immune reconstitution. Thus, HEV RT-PCR testing is the preferred diagnostic method in these immunocompromised patients. Treatment of HEV infection after transplantation includes reduction of immunosuppressive therapy, while there is no registered drug therapy. Anecdotal evidence supports the use of oral ribavirin in immunocompromised patients. In our study, three patients cleared HEV with a dose reduction of immunosuppressive agents (i.e. cyclosporine A and/or prednisone) alone. Treatment with ribavirin should be considered in patients, for whom immunosuppression cannot be reduced, such as, for example, patients with active GVHD. The optimal daily dose of ribavirin is unknown, in case reports sustained viral response has been described with daily dosages between 200 mg and 1200 mg.[80, 89] If HEV infection is confirmed prior to alloHSCT, it can be considered as a contraindication to transplantation. Clearance of HEV viremia

is therefore of high importance. AlloHSCT candidates are usually pre-treated with chemotherapy, resulting in impaired and/or delayed immune reconstitution. Therefore, early ribavirin treatment can be initiated to support rapid HEV clearance in these future alloHSCT recipients.

In conclusion, this study shows that recipients of alloHSCT are at risk for HEV infection, albeit with a relatively low risk. However, the probability of developing severe chronic hepatitis in immunocompromised patients is high. Therefore, patients should be screened for HEV antibodies and HEV RNA prior to alloHSCT, and patients with acute liver enzyme abnormalities after alloHSCT should be analyzed for HEV reactivation and/or infection. Moreover, HEV should be included in the differential diagnosis of liver GVHD and drug induced liver injury, because of the largely overlapping picture with respect to liver enzyme abnormalities.

ACKNOWLEDGEMENTS

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Chapter 3.4

Hepatitis E virus infection in a tertiary referral center in the Netherlands

Clinical relevance and impact on patient morbidity and mortality

Charlotte M. Nijskens, **Suzan D. Pas**, Jan Cornelissen, Kadir Caliskan, Rogier A.S. Hoek, Dennis A. Hesselink, Annemiek A. van der Eijk , Robert A. de Man

submitted

ABSTRACT

Background

Autochthonous Hepatitis E virus (HEV) infections can have important clinical consequences in patients with pre-existent (liver) disease and immunocompromised patients. To evaluate patients at risk and optimize treatment strategies, we studied the clinical course and treatment outcome in patients diagnosed with HEV viremia in our hospital.

Methodology

Between January 2008 and March 2015 we included all patients with HEV genotype 3 (HEV gt 3) infections diagnosed by means of quantitative real-time reverse transcription-polymerase chain reaction test (RT-PCR). Clinical data were evaluated retrospectively.

Results

In total 79 patients were included. Forty-nine patients (62%) were male, median age of all patients was 52 years (range 13 - 79). Sixty-one (77%) patients were immunocompromised. Three patients (3.8%) had only transient viremia, forty-three (54.5%)) cleared the infection within six months and twenty-six (32.9%) developed chronic hepatitis. Five patients (6,3%) were lost to follow-up. All patients developing chronic hepatitis were immunocompromised. Overall, thirteen (16%) patients within this cohort died. Three patients had pre-existent liver diseases and died of liver-related causes. Time between diagnosis and death was shorter for patients with pre-existent liver diseases ($p = 0.03$). Twenty-eight percent of patients on immunosuppressive medication achieved viral clearance after reducing the dose of immunosuppressive therapy. Thirty patients (38.0%) were treated with off-label ribavirin in which 25 (83.3%) a sustained viral response has been documented. Median time from the start of ribavirin to HEV clearance was two months.

Conclusion

Autochthonous HEV viremia mainly presents in patients with underlying chronic liver diseases or an impaired immune system. Patients with pre-existent liver diseases who acquire an HEV infection are at high risk for complications and even death. The off-label use of ribavirin can cure HEV infection.

INTRODUCTION

Hepatitis E virus (HEV) is a positive sense, single stranded, non-enveloped RNA virus, discovered in the early 1980s. [3] The virus consists of 4 genotypes that can infect humans, all with a distinct geographical distribution. HEV genotype (gt) 1 and 2 are found in developing countries in Asia and Africa, where they can cause large outbreaks of acute hepatitis via the fecal-oral route. Especially pregnant women are at risk of a fulminant course. [148] In developed countries, HEV gt 3 and 4 are mainly transmitted from animal reservoirs. HEV gt 3 is responsible for the autochthonous HEV infections in Europe, gt 4 is mainly found in Asia. Pigs and wild boar are thought to have the greatest contribution to the transmission of gt 3. [149] However recently, cases regarding transfusion-transmitted HEV gt 3 infections have also been reported. [150, 151] About one in two patients infected with gt 1 and gt 2 develop clinical symptoms whereas 67 – 98% of patients infected with gt 3 and gt 4 remain asymptomatic. [152] The clinical features of acute HEV infection caused by the different genotypes cannot be distinguished from each other and range from transient and asymptomatic viremia to acute hepatitis with malaise, jaundice and even liver failure. [68, 153, 154] The fulminant course of the infection during pregnancy is only seen in women infected with HEV gt 1.

Chronic HEV gt 3 infections are rarely seen in otherwise healthy individuals but are increasingly being reported in immunocompromised patients. Patients receiving solid-organ transplantations (SOT) that require life-long immunosuppressive therapy to prevent graft rejection and patients with haematological malignancies, are prone to develop chronic HEV gt 3.[155, 156] In these patients chronic HEV infections can progress to fibrosis [89] and even cirrhosis [71] which occasionally requires liver transplantation. Chronic HEV gt 4 infections are sporadically reported [157] whereas chronic infections of HEV gt 1 and HEV gt 2 do not occur.

In immunocompetent patients, an acute HEV infection normally does not require treatment. Given the strong association between the use of immunosuppressive drugs and chronic HEV, dose reduction or even withdrawal of immunosuppression if possible, is considered to be the first step in the treatment of HEV infections. [71] In patients who fail to eliminate the virus after reduction of immunosuppressive drugs or whose dose of immunosuppressive drugs cannot be reduced, antiviral therapy should be considered. Antiviral therapy consists of the off-label use of pegylated interferon alpha or ribavirin therapy, or a combination of both. [127, 130, 158]

Studies evaluating the clinical course of HEV gt 3 infection mainly focus on patients with SOT, haematological malignancies and HIV. We studied the clinical course in all patients diagnosed with HEV gt 3 viremia in our hospital to define clinical outcome, therapeutic interventions and treatment effect.

METHODS

Sample collection

This retrospective cohort-study analysis was conducted at the Erasmus MC, University Medical Center Rotterdam, a tertiary referral and transplant center in The Netherlands. We included all patients who tested positive for HEV RNA genotype 3 in serum or EDTA-plasma at the Department of Viroscience between January 2008 and March 2015.

Patients were tested prospectively for HEV RNA during yearly routine checkup after transplantation or in case of unexplained elevated liver enzymes. In rare cases, patients were referred from other hospitals because of unexplained elevated liver enzymes or for treatment of the HEV infection. In our hospital, liver, lung and heart transplant recipients are routinely screened for HEV infections once prior to transplantation and annually thereafter. Patients with haematological malignancies who receive an allogeneic stem cell transplantation (alloHSCT) are also screened prior to the transplantation and in case of unexplained elevated liver enzymes. At present, kidney transplant recipients are not routinely screened for HEV.

Patients were included if they tested positive for HEV gt 3 RNA in serum or blood, regardless of the HEV RNA level and anti HEV IgM or IgG status. If available, stored blood samples were tested to trace the first positive sample. Patients were excluded if the infection was caused by a genotype other than HEV gt 3. Patient's baseline demographics were obtained, including sex, age, comorbidities, type of medication and biochemistry and virological laboratory results at the time of diagnosis. Based on these data, we classified the patients as immunocompetent or immunocompromised. Patients were immunocompromised if they met one of the following criteria: 1) use of immunosuppressive medication after transplantation; 2) use of biologicals in case of rheumatoid arthritis or inflammatory bowel disease; 3) treatment by a course of or continuous chemotherapy during the last three months; 4) presence of haematological malignancy or primary immunodeficiency. Thereafter we evaluated the medical records from recruited patients to explore

the clinical course, duration and outcome of the infection, laboratory results at peak of infection and at the end of follow-up, and therapeutic interventions. If available, data were collected concerning causes of death. Causes of death were evaluated by two authors independently without conflicting results.
Ethical approval was given by the Medical Ethical Review Board of Erasmus MC.

Virological parameters
Serum was tested for HEV RNA by means of an internally controlled quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR), as described previously [90]. The RT-PCR has a lower limit of detection (95% hit rate) of 143 IU/ml as determined by the first World Health Organization standard for HEV RNA nucleic acid amplification testing-based assays.

Clinical outcome
In all patients we evaluated the clinical outcome of the infection: transient viremia, acute hepatitis and chronic hepatitis. Mortality due to liver disease or other causes was a secondary outcome parameter. Transient viremia was defined as the presence of HEV RNA in blood without ALT elevation. Acute hepatitis was defined as the presence of HEV viremia for less than six months in combination with an elevation of alanine aminotransferase (ALT) above the normal limit of 44 U/l. If HEV RNA in serum was detectable for at least six months patients were said to have chronic hepatitis. End of infection was stated on the first day RT-PCR was negative. Liver-related mortality was defined as death related to liver failure or liver-related complications. In patients treated with antiviral therapy, an undetectable HEV RNA load at the end of treatment was defined as end of treatment response (ETR) whereas undetectable HEV RNA for at least 3 months post treatment was defined as sustained viral response (SVR).

Data analysis
Statistical analysis was performed using SPSS version 21 (IBM The Netherlands). Within each clinical outcome baseline characteristics of patients were compared. To analyze continuous parameters with a normal distribution, Student's *t* test was used. For categorical or dichotomized parameters, proportions between groups were compared using Chi squared test or Fisher's exact test. A p-value lower than 0.05, was considered statistically significant.

RESULTS

A total of 80 patients tested positive for HEV RNA in serum. In all but one patient HEV gt 3 was detected so a total of 79 patients were included. Thirty-two patients (40.5%) were tested in the context of screening before or after transplantation, 5 (6.3%) were suspected of graft versus host disease (GvHD) and 42 (53.2%) were tested in case of unexplained hepatitis. Forty-nine patients were male (62.0%) and median age was 52 years (13 – 79). In total 61 patients (77.2%) were immunocompromised and 3 patients (3.8%) used low dose prednisone (n=2) or low dose methotrexate (n=1) (Table 1).

Thirty-eight patients (48.1%) were SOT-recipients of which all but one used immunosuppressive medication, with the following type of SOT: liver (n= 16), heart (n=13), kidney (n=5), lung (n=1), liver and kidney (n=1), kidney and pancreas (n=1), heart and lung (n=1). Fifty percent of SOT-recipients were diagnosed with HEV viremia within 800 days after transplantation (range 7 – 7304 days). Twelve patients (15.2%) had a history of alloHSCT. The details on immunosuppressive treatment are displayed in Figure 1. Nineteen patients (24.1%) had a pre-existent, non-HEV related active liver disease at moment of diagnosis.

Table 1 Patient characteristics

Characteristic	
Sex	
Male, number	49 (62.0%)
Female, number	30 (38.0%)
Age at onset of infection in years	
Median (range)	52 (13 – 79)
BMI kg/m²	
Median (range)	26.6 (18.3 - 39.9)
Immunocompromised	
Number	61 (77.2%)
HEV RNA-concentration at presentation IU/ml	
Median (range)	6.6E4 (<143 – 1.7E8)
ALAT, presentation U/l	
Median (range)	85 (15 – 4801)
ASAT, presentation U/l	
Median (range)	75 (17 – 1845)
ALAT, peak U/l	
Median (range)	209 (26 – 4801)
ASAT, peak U/l	
Median (range)	185 (26 – 1848)
Follow up time in days	
Median (range)	131 (1 – 2328)

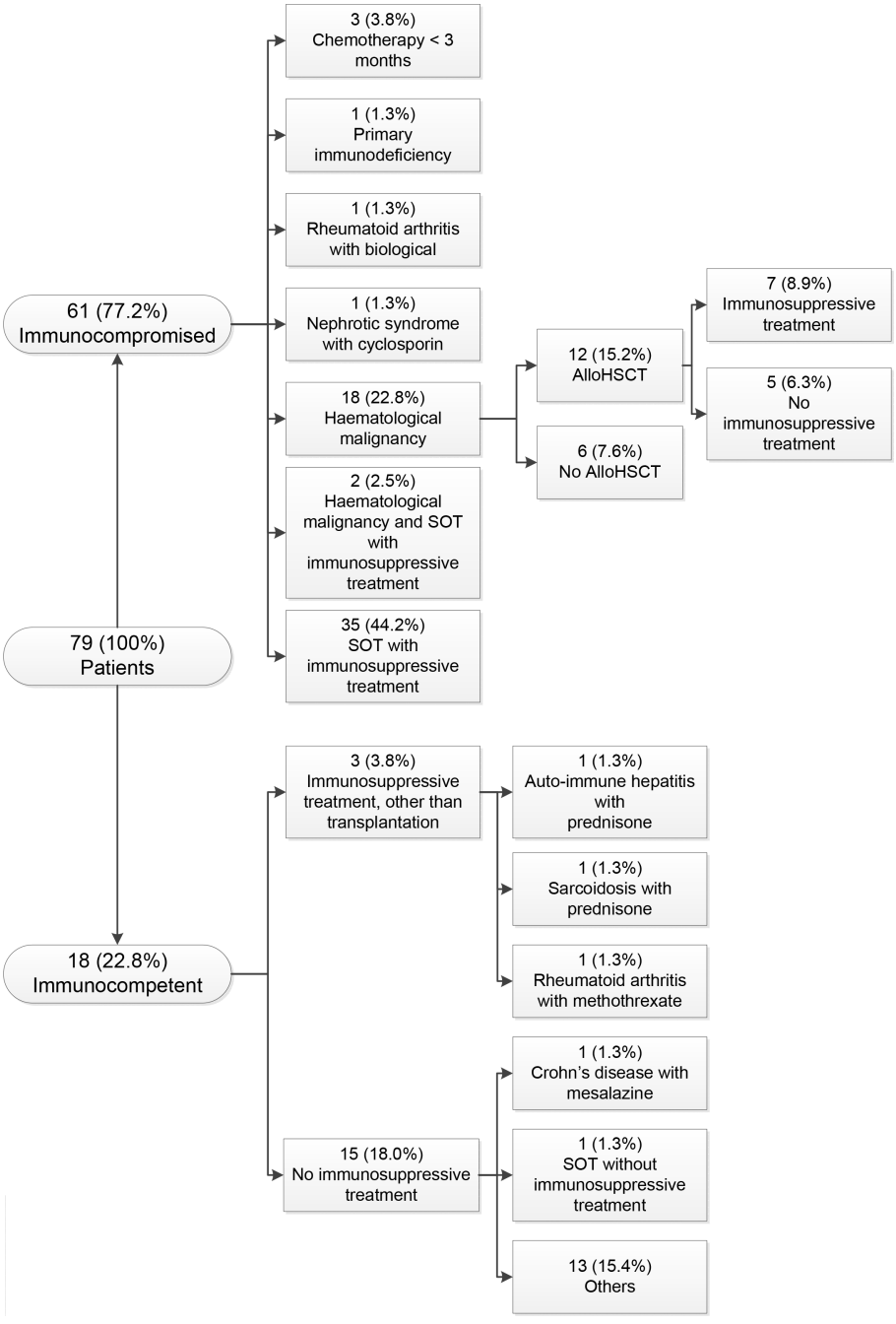


Figure 1 Causes of immunosuppression and use of immunosuppressive treatment

Clinical course of infection

In total, 3 patients (3.8%) had only transient viremia with HEV RNA levels around the lower detection rate of 143 IU/ml. Two patients were heart transplant recipients and treated with immunosuppressive medication. They were infected more than six months after transplantation which made infection by transplantation unlikely given the incubation period of 2-6 weeks for HEV. The other patient was known with alcoholic liver cirrhosis and screened prior to liver transplantation by which transient viremia was detected.

Forty-three patients (54.5%) had signs of acute hepatitis and cleared the infection within six months and 26 patients (32.9%) developed chronic hepatitis. 5 patients (6.3%) were lost to follow up after having signs of acute hepatitis. In these patients we could not determine whether they developed chronic hepatitis or not. (Figure 2)

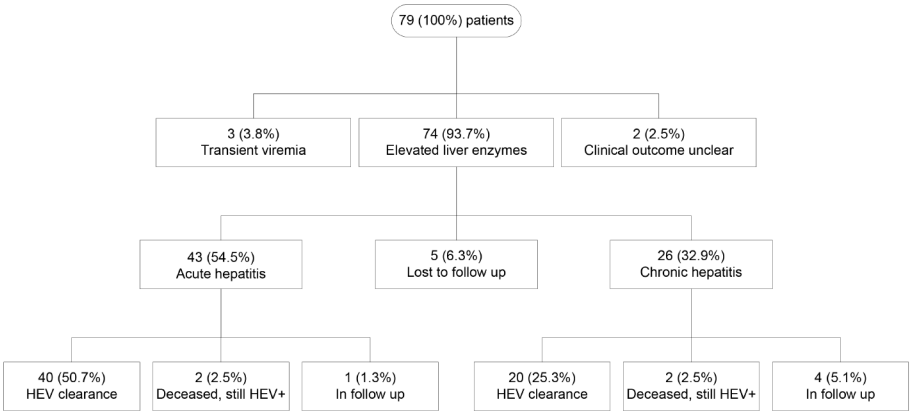


Figure 2 Flowchart of the clinical course in HEV-infected patients of the cohort

Table 2 **Patient characteristics of acute versus chronic hepatitis E**

Characteristic	Acute (n = 43)	Chronic (n = 26)	P-value
Sex			
Male, number	28 (65.1%)	17 (65.4%)	1.0
Female, number	15 (34.9%)	9 (34.6%)	
Age at onset of infection in years			
Median (range)	53 (16 - 67)	54 (40 - 60)	0.58
Immunocompromised, number	28 (65.1%)	26 (100%)	0.001
Solid organ transplantation, number	16 (37.2%)	18 (69.2%)	0.01
Liver (including 1 Liver + Kidney)	11 (25.6%)	5 (19.2%)	0.55
Heart (including 1 Heart + Lung)	2 (4.7%)	9 (34.6%)	0.002
Kidney (including 1 Liver + Kidney)	4 (9.3%)	3 (11.5%)	1.0
Lung (including 1 Heart + Lung)	0	2 (7.7%)	0.14
AlloHSCT	5 (11.6%)	7 (26.9%)	0.19
Immunosuppressive treatment after transplantation	17 (39.5%)	24 (92.3%)	<0.001

All patients who developed chronic hepatitis were immunocompromised whereas 65.1% of the patients with an acute hepatitis were immunocompromised (p = 0.001). (Table 2) We found a significant difference in the prevalence of SOT in patients developing chronic hepatitis compared to acute hepatitis: 69.2% vs. 37.2% respectively (p = 0.01). This was linked to the use of immunosuppressive therapy after transplantation (92.3% vs. 39.5%, p < 0.001). Especially heart transplant recipients significantly more often developed chronic hepatitis (p= 0.002). The 9 heart transplant recipients who developed chronic hepatitis were treated with tacrolimus-based therapy whereas the only two patients with transient viremia had cyclosporine-based therapy. We found no significant difference in outcome in patients who had pre-existent, active liver diseases or who were immunocompromised due to haematological malignancies.

In 2 patients the clinical outcome could not be determined. One patient was screened for HEV after liver transplantation annually. At the moment of diagnosis no laboratory findings indicating hepatitis were present. One year later, qRT-PCR was repeated and negative. One heart transplant recipient was screened only once and did not have any follow up.

Deceased patients

Eventually 13 patients died, of which 4 were HEV RNA positive and 8 had cleared the virus at time of death. In 1 patient, the HEV status at the time of death could not be determined.

The four patients who were viremic at the time of death all had haematological malignancies and died of sepsis due to respiratory infections (n=3) and meningitis (n=1) (51, 53, 198 and 393 days after HEV infection). None of these patients was treated for the HEV infection.

Eight patients died after clearing the infection. Three of these patients had pre-existent liver diseases with cirrhosis. They died shortly after clearing the infection, i.e. after 4, 17 and 54 days. One died of liver decompensation with multi-organ failure, one of complications due to spontaneous bacterial peritonitis and the third patient died of renal failure. The other five patients who died after clearing the virus were immunocompromised due to haematological malignancies or immunosuppressive therapy.

When comparing time from diagnosis to death between patients with and without pre-existent liver disease, we found a significantly shorter time for patients with cirrhosis (median 35 vs. 596 days; $p = 0.028$). This suggests that a HEV infection triggers acute on chronic liver disease with mortality in patients with pre-existent (decompensated) liver disease.

One heart transplant recipient died of renal failure, 827 days after the HEV infection was diagnosed. Because the patient died in a nursery home, no sufficient follow up was present to determine whether he was HEV-positive at moment of death or not.

Immunocompetent patients

Eighteen patients were immunocompetent. One patient had only transient viremia, 15 patients had acute hepatitis and 2 were lost to follow up. Eleven out of the 15 patients had spontaneous resolving hepatitis and 4 were successfully treated with ribavirin of whom three patients had underlying diseases. One patient had liver cirrhosis and hepatocellular carcinoma, one patient had auto-immune hepatitis and the third patient had short bowel disease due to Crohn's Disease. The fourth patient treated with ribavirin had no underlying disease but did have signs of non-resolving, imminent liver failure. After two days of ribavirin treatment (600 mg b.i.d.), HEV RNA became undetectable.

Immunocompromised patients

Sixty-one patients were immunocompromised. Of these patients, 27 were not treated. They were immunocompromised due to chemotherapy (n=8), use of immunosuppressive drugs (n=16), haematological malignancies (n=2) and common variable immune deficiency treated with immunoglobulin substitution (n=1). Two patients had transient viremia, 14 acute hepatitis, 4 chronic hepatitis, 2 were lost to follow up and 5 died while HEV RNA positive. SOT-patients who were not treated did not clear the infection in month 3 to 6 after diagnosis suggesting that the diagnosis of chronic HEV infection can be made after 3 months of HEV viremia.

Reduction of immunosuppressive drugs

Eight patients were treated solely by reducing their immunosuppressive drug burden. Five patients used immunosuppressive drugs after transplantation. In 4 out of these 5 patients the calcineurin-inhibitor was adjusted with 50% dose reduction and in the fifth patient sirolimus was stopped. Prednisone was stopped in 3 out of these 5 patients. The other three patients used immunosuppressive drugs for other indications. In one patient mycophenolic acid for nephrotic syndrome was stopped and dose of cyclosporine was halved. In the second patient adalimumab and methotrexate for rheumatoid arthritis were temporarily stopped. The third patient, with a history of polycythemia vera, was treated with a temporary stop of hydroxycarbamide. All patients successfully cleared the virus, within a median of 207 days (27 - 1306).

Ribavirin treatment

In total 30 patients were treated with ribavirin, starting a median of 97 days (0 - 1825) after the diagnosis of HEV infection (Table 3*).* Ribavirin was started at the discretion of the treating physician. Thirteen patients started within less than 3 months after diagnosis, 6 within 3-4 months and 11 patients more than six months after moment of diagnosis. Median dose administered was 800 mg/day, i.e. 10.4 mg/kg (1.96 - 25.04) and median duration of treatment was 94 days (10 - 560). Eighty-seven percent of treated patients were immunocompromised whereas 76% in the non-treatment group were immunocompromised ($p = 0.036$) due to a significantly higher prevalence of SOT and use of immunosuppressive drugs after transplantation. When comparing these groups, other causes for a suppressed immune system were equally distributed between treatment and non-treatment groups. In 10 patients the dose of immunosuppressive therapy was reduced a median of 45 days (10 – 365) prior to the start of ribavirin treatment.

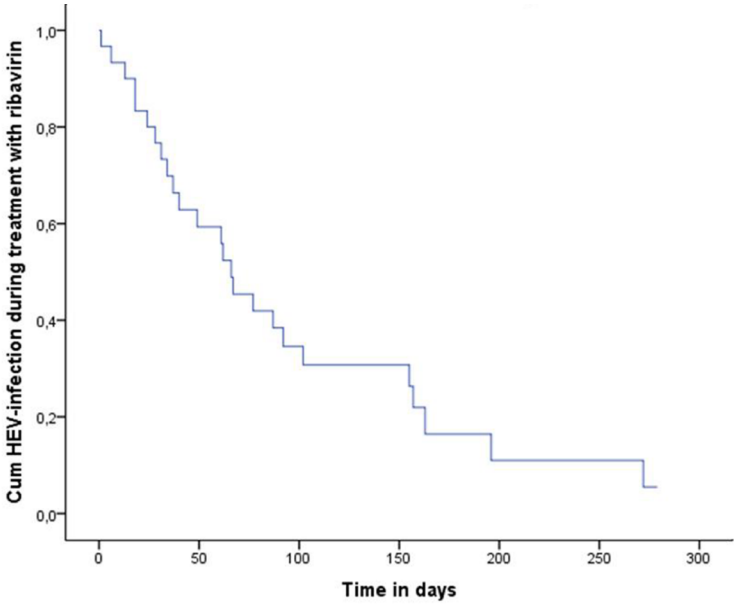
In 11 patients, ribavirin treatment was started concomitantly with reduction of the immunosuppressive therapy.

Table 3 Patient characteristics of patients treated with ribavirin

Characteristic	number (%)
Sex	
Male	19 (63.3%)
Female	11 (36.7%)
Immunocompromised	26 (86.7%)
Solid organ transplantation	19 (63.3%)
Liver	8 (26.7%)
Heart (including Heart + Lung)	9 (30.0%)
Kidney	2 (6.7%)
AlloHSCT	4 (13.3%)
Immunosuppressive treatment after transplantation	22 (73.3%)

Eighty-three percent of patients (25/30) achieved SVR. Seven patients had a rapid viral response (< 5 weeks from start ribavirin), 10 patients had an early viral response (< 13 weeks) and 8 patients had a late viral response (> 13 weeks). (Figure 3) Median time until HEV clearance was 61 days (1 – 272). Four patients were still being treated at end of follow up (33, 91, 102 and 161 days of treatment). Three of these patients reached negative RT-PCR but had no sufficient follow up yet to ascertain SVR. In one patient treatment was interrupted because of incurable oropharyngeal carcinoma. One patient was treated for only 10 days because of respiratory syncytial virus and had a late viral response after 272 days.
Main adverse events were anemia, anorexia and affective disorders. In only one patient this caused a shortening of treatment by three weeks.

Among the 30 patients who were treated with ribavirin, only one patient had recurrence. She initially responded to ribavirin after treatment with 800 mg/day for 90 days. No HEV RNA was detected in serum. After three months, RT-PCR was positive again with rising viral loads. Phylogenetic analysis was performed with concatenated ORF1 and ORF2 sequences, which revealed 9 nucleotide changes in 810bp hypervariable regions of the HEV genome. After one year, she was retreated successfully with ribavirin 800 mg/day for 120 days.



Time in days	0	50	100	150	200	250	300
Patients under observation	30	17	9	7	2	2	0
Patients who cleared infection	0	12	19	20	24	24	25

Figure 3 Time to HEV clearance (negative qualitative RT-PCR) in patients treated with ribavirin. Treatment was started at day 0.

DISCUSSION

We describe the clinical characteristics and outcome of HEV gt 3 infections in a tertiary referral hospital based population in the Netherlands and show that HEV can cause significant morbidity and contribute to mortality in patients. The off label use of ribavirin therapy provides viral clearance within a median of two months. To the best of our knowledge this retrospective single center study is the largest series so far to evaluate the clinical course and treatment outcome of HEV infection in Western Europe.

Our study showed a predilection for middle-aged men which is in line with several studies concerning autochthonous HEV infections. [68, 159] Patients in these studies presented at a median age of around 60 years with male to female ratios of 1:3. [68, 153, 159, 160] Previously it was thought that middle-aged men were more often exposed to HEV than women. However, recent screening of blood donors

in The Netherlands showed no difference in seroprevalence of HEV between men and women, which makes an equal exposure to the virus more likely. [161] An explanation for the higher incidence of symptomatic hepatitis among middle-aged, non-transplant patients may be that these patients more often have significant co-morbidities than young individuals and that these comorbidities predispose for symptomatic HEV. Previous studies also found that excessive alcohol consumption (> 22 units of alcohol/week), especially present in men, contributes to the risk of having clinical symptoms. [162, 163] However, we were not able to reliably evaluate the alcohol consumption in our patients.

Our study confirms the results of previous studies, in which chronic HEV infections are solely found in immunocompromised patients, mostly SOT-recipients. [69, 71] Due to the impaired immune system the virus can persist in the body for more than six months. [164] Moreover, some cases of chronic HEV have been reported in patients with HIV [165] and haematological malignancies[166, 167]. In our study seventy-five percent were SOT-recipients and 25% had a haematological malignancy. Especially heart transplant recipients were prone to develop chronic hepatitis. When comparing their medication, we found that all heart transplant recipients who developed chronic HEV were treated with tacrolimus-based immunosuppressive therapy. This was also seen in lung transplant recipients. Kamar *et al*. described earlier that the use of tacrolimus rather than cyclosporine A attributes to the risk of chronic HEV infections. This may be due to a greater down-regulation of the T-cell response against the virus. [71] Likewise, in our study the only two heart transplant recipients who had transient viremia were treated with cyclosporine A.

Several studies revealed that approximately 60% of SOT-recipients develop chronic infection. [71, 76, 168] In our cohort, 47% of SOT-recipients developed a chronic infection. This slight difference can be explained by two reasons. First, six out of 38 patients were successfully treated with ribavirin within six months after diagnosis. Hence, no evolution of the disease to chronicity was awaited. Second, two out of 38 patients had no sufficient follow up so a chronic infection cannot be excluded.

In immunocompetent patients, HEV gt 3 rarely causes a symptomatic hepatitis but it can result in acute fulminant hepatitis with decompensation in patients with underlying chronic liver disease. [154, 169] Although studied among limited numbers, high mortality rates up to 70% in these patients have been reported.

[154] In the present study, four immunocompromised patients died while still HEV RNA positive. Moreover, three patients with pre-existent cirrhosis died shortly after clearing the virus due to liver-related causes. The time from diagnosis to death was significantly shorter when compared to patients without underlying liver diseases. In patients with pre-existent cirrhosis, the superinfection with HEV probably provokes such an inflammatory response that, although leading to clearance, causes additional damage to the liver. Eventually, the liver cannot recover and death may follow.

Kamar *et al*. evaluated the effect of dose reduction in immunosuppressive therapy among SOT-recipients to achieve HEV clearance. [71] During the first 6-month period after diagnosis, the daily dose of immunosuppressive treatment was reduced in 56 patients. Eighteen patients (32.1%) achieved HEV clearance with a median of 19.5 months after diagnosis. In our study 29 patients had a reduction in dose of immunosuppressive treatment and 8 (27.6%) achieved viral clearance. Although in two patients immunosuppressive drugs were used for other reasons than prevention of rejection, these drugs have considerable immunosuppressive properties. One patient used hydroxycarbamide, an inhibitor of ribonucleotide reductase, that can cause bone marrow suppression and leucopenia. [170] The other patient used adalimumab, an anti-tumor-necrosis-factor-alpha agent with a strong immunosuppressive action that provides a high risk for serious infections. [171]

In patients with persisting HEV infections who fail to eliminate the virus after reduction of immunosuppressive drugs or whose dose of immunosuppressive drugs cannot be reduced, specific antiviral therapies are required. Complete viral clearance has been described for the use of pegylated interferon alpha. It is shown to be effective in patients with liver transplantation [127] and in one kidney-transplant recipient [126]. However, interferon therapy is contraindicated in SOT-recipients due to the increased risk of acute rejection. In these patients, the off-label use of ribavirin has shown good results in small case series and in one larger case series consisting of 59 patients. [158] Kamar *et al*. found SVR in 85% of patients treated with ribavirin. They suggest that a treatment duration of 3 months is sufficient to achieve SVR. However, in patients with persistent replication at 1 month and in patients with recurrence, a longer period of treatment may be needed. [158] In our cohort, 30 patients were treated with ribavirin of which 87% were immunocompromised. Eighty-three percent of patients were successfully treated with ribavirin for a median of 94 days (10 – 560) and 10% had a negative

qRT-PCR but no sufficient follow up yet to determine SVR. We found not only a successful response in SOT-patients but also in three patients with haematological malignancies. Median treatment duration to HEV clearance was two months which demonstrates a rapid response after the start of treatment. Only one patient had recurrence but achieved viral clearance after retreatment with ribavirin. In this study, thirteen patients were treated after having been infected with HEV for less than three months. We cannot exclude that (some of) these patients would have had spontaneous clearance without ribavirin treatment. In SOT-recipients it was found by Kamar *et al*. that if HEV RNA persists for more than three months, no spontaneous clearance will be observed between months 3 and 6 after infection. [172] This is in line with our finding that SOT-patients did not have spontaneous HEV clearance in month 3 to 6. We therefore recommend to start ribavirin treatment after three months in SOT-patients to prevent a chronic course with potential adverse events.

Due to the retrospective design of our study, we came across some limitations. First and foremost, some patients did not have sufficient or frequent follow up. Hereby the exact date of viral clearance could not always be determined. Second, the doses and durations of ribavirin altered during the last years.

Chapter 4

Hepatitis E virus genotype 3 infection of human liver chimeric mice and A549 cells as models for chronic HEV infection

Martijn D.B. van de Garde, **Suzan D. Pas**, Guido van der Net, Albert D.M.E. Osterhaus, Bart L. Haagmans, Andre Boonstra, Thomas Vanwolleghem

submitted

ABSTRACT

Background & Aims

Genotype (gt) 3 hepatitis E virus (HEV) infections are emerging in Western countries. Partly due to the lack of suitable model systems, their transmission, infectivity and pathogenesis are poorly understood.

Methods

Here we characterize the *in vitro* and *in vivo* infectivity of HEV RNA positive EDTA-plasma, feces or liver-biopsy samples from 8 immunocompromised patients with chronic gt 3 HEV in the A549 cell culture system and in a human-liver chimeric mouse model (uPA$^{+/+}$Nod-SCID-IL2R$\gamma^{-/-}$).

Results

HEV antibody negative EDTA-plasma samples showed no or slower propagation within 2 weeks after inoculation on A549 cells, compared to feces- or liver-derived inocula of similar HEV RNA content. To corroborate these infectivity differences, selected samples were i.v. inoculated in human-liver chimeric mice. HEV RNA levels up to 8 log IU HEV RNA/gram, were consistently present in 100% of chimeric mouse livers from week 2-14 after inoculation with human feces-derived HEV. HEV RNA was detected in feces of these mice, while HEV viremia was low and inconsistently present. A human liver biopsy-derived HEV inoculum resulted in moderate to high HEV RNA levels in mouse feces, bile and liver after infection. In contrast, seronegative, HEV RNA-positive EDTA-plasma was not infectious in any of the inoculated animals.

Conclusion

Infectivity of feces-derived human HEV is higher compared to EDTA-plasma derived HEV both *in vitro* and *in vivo*. Persistent HEV gt 3 infections in chimeric mice, show preferential viral shedding towards mouse bile and feces, mimicking the course of infection in humans.

INTRODUCTION

Emerging hepatitis E virus (HEV) genotype (gt) 3 infections are reported in western countries, including France, the United Kingdom and the Netherlands with increasing frequency [173-175]. While reports showed an overall decrease in anti-HEV seroprevalence from 1996 to 2011, young adult blood donors demonstrated higher seroprevalences from 2000 to 2011 [176, 177]. In addition HEV RNA positive blood donations were reported to increase in the Netherlands since 2012 [178]. Although the exact source of this HEV gt 3 endemic is unknown, it is likely that domestic swine and pig farming plays a critical role. HEV RNA gt 3 was detected in approximately half of the pig farms in the Netherlands [179].

HEV is a non-enveloped positive-sense single-stranded RNA virus of the genus *Orthohepevirus* and family *Hepeviridae* [180]. Four major HEV genotypes infecting humans have been described so far. Genotype 1 and 2 strains are only isolated from humans, whereas gt 3 and gt 4 strains are considered zoonotic viruses, present in both humans and several other species like pigs and wild game. HEV is spread through the oral-fecal route via contaminated water in developing countries or amongst others, via direct contact with animals or the consumption of undercooked meat in industrialized countries. In immunocompetent individuals, HEV infection is self-limiting, often asymptomatic and thus remains largely underdiagnosed. HEV infections in immunosuppressed patients, such as solid-organ transplant recipients, often persist and can progress quickly to liver fibrosis and cirrhosis [89, 181, 182].

The pathogenesis of HEV infections is poorly understood in part due to a lack of suitable *in vitro* and *in vivo* model systems**.** Antiviral treatment options for chronic HEV infections in solid organ transplant recipients are limited to ribavirin, but no randomized controlled trials have prospectively examined the optimal dose, duration and monitoring frequency [158]. In addition, no adequate animal model exists for the observed chronicity rates of HEV in immunocompromised patients, nor do the existing natural animal hosts develop overt clinical signs of hepatitis [183].

In this study, we utilize the *in vitro* A549 cell culture system and the established humanized liver urokinase-type plasminogen activator (uPA)-transgenic mouse model on a severely immunodeficient NOD/Shi-*scid*/IL-2Rγ^{null} background (uPA-NOG) to explore the infectivity of HEV gt 3 samples of different clinical origins [90, 156, 184, 185]. We demonstrate that *in vitro* and *in vivo* infectivity is much lower for human EDTA-plasma, compared to feces- and liver- derived inocula. Once infected, persistent intrahepatic viral replication is seen in all chimeric mice, with

preferential viral shedding to mouse bile and feces, reminiscent of human HEV infections. Chimeric human liver mice are therefore a suitable model for future studies on HEV infectivity, pathogenesis and antiviral efficacy.

MATERIAL AND METHODS

Inoculum preparation

Inocula were obtained from heart- (n=4), liver- (n=1), and allogeneic hematopoietic stem cell (n=2) transplant recipients and one recipient who received both heart and kidney grafts (see Table 1), treated either at the University Hospital Antwerp or the Erasmus Medical Center [90, 156]. All had detectable HEV RNA in their EDTA-plasma for more than 6 months (defined as chronic HEV infection). Inocula used for infection had no detectable levels of anti-HEV IgM and IgG antibodies according to the results obtained with a commercially available ELISA (Wantai, Beijing, China). Open reading frame 1 and 2 sequences of the inocula are available at genbank (Table 1). Clinical sequelae have been described elsewhere [89, 90, 156]. Fecal suspensions were prepared as follows: 3 gram feces was vortexed thoroughly in 10 ml saline and centrifuged (450g, 3 min). After 2 additional centrifugation steps (14000g, 5 min), the supernatant was passed through a 0.45 µm filter. A cryopreserved liver biopsy fragment from one heart transplant patient was homogenized in 500 µl saline using ceramic beads. The supernatant was used as inoculum after centrifugation (5000g, 10 min). All inocula were kept at -80°C until use.

Table 1 Inocula of chronic HEV gt 3 patients for in vitro and in vivo infection

Case no.	1st year HEV+	age @ HEV+	Sex	Morbidity#	Genbank accession ORF1	Genbank accession ORF2	HEV RNA (log IU/ml)			reference
							serum	feces	liver	
HEV0008	2008	55	F	AlloHSCT	JQ015407	KT198654		7.00	-	[156]
HEV0014*	2011	40	F	AlloHSCT	KC171436	KP895853	6.84*	6.77	-	[156]
HEV0033	2010	51	M	HTx	JQ015427	KT198656		8.67	-	[89, 90]
HEV0047	2010	56	M	HTx	JQ015425	KT198657		7.01	-	[89, 90]
HEV0063	2010	19	M	LTX	JQ015426	KT198658		8.04		[90]
HEV0069*	2010	62	M	HTx	JQ015423	KP895854	7.30	8.80*	-	[89, 90]
HEV0081	2009	50	M	HTx + KTx	JQ015418	KT198659		7.95	-	[89, 90]
HEV0122*	2014	63	M	HTX	KP895856	KP895855	6.74*	8.80*	6.26*	this publication

*****Inocula used for both *in vivo and in vitro* infection

[#] AlloHSCT allogeneic hematopoietic stem cell transplantation; HTx heart transplantation; LTx liver transplantation; KTx kidney transplantation

Hepatitis E virus propagation

Human adenocarcinoma alveolar basal epithelial cells (A549) were seeded on a coverslip in 24-well plate, in A549 growth medium containing Dulbecco's modified Eagles Medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS, Greiner Bio-one, Kremsmünster, Austria), 0.08% $NaHCO_3$, 2 mM L-glutamine (Lonza), 1% penicillin/streptomycin (pen/strep, Lonza) and 0.5 µg/µl amphotericin B (Pharmacy, ErasmusMC, Rotterdam, The Netherlands). Three days after seeding, cells were washed once with phosphate buffered saline (PBS, Oxoid, Hampshire, UK) and inoculated with either HEV derived from different sample types or mock and incubated for 1 hour at 36.5°C in a humidified 5% CO_2 incubator. Liver-derived samples were diluted 1:10 prior to inoculation, to dilute toxic substances. The virus suspension was then removed and cells were washed three times with PBS before adding maintenance medium, containing 1:1 mixture of DMEM (Lonza)/Ham's F-12 (Life technologies), supplemented with 2% FBS, 20 mM HEPES (Lonza), 0.4% $NaHCO_3$, 2 mM L-glutamine, 0.3% Bovine Albumin Fraction V (BSA, Lonza), 1% pen/strep and 2.5 µg/µl amphotericin B and incubated at 36.5°C in a humidified 5% CO_2 incubator. Proper washing was documented by the absence of HEV RNA (Ct>38) in the last PBS supernatant. For monitoring virus propagation, every two to three days cells were inspected on cytophathogenic effect (CPE) and viability, culture medium was refreshed with maintenance medium (1:1) and supernatant was taken for HEV qPCR. Supernatant was passaged onto naïve A549 cells 15-20 days after infection, after which the same routine was used.

To test the 50% tissue culture infectious dose ($TCID_{50}$), DMEM-adapted A549 cells (as documented above) were cultured in a flat bottom 96 wells plate. HEV virus stock (positive control) and samples were then 10x serial diluted in quattro-plo (in a separate round bottom 96-wells plate). Subsequently, 100 µl virus suspension or mock were inoculated for 1 hour at 36.5°C in a 5% CO_2 humidified incubator. After washing three times with PBS and addition of maintenance medium, the virus was allowed to replicate for 7 days, after which replication was read by qualitative HEV qPCR. $TCID_{50}$ is calculated according to Spearman/Karber.

Mouse origin and genotyping

uPA-NOG mice were kindly provided by the Central Institute for Experimental Animals (Kawasaki, Japan) [184]. Mice were bred at the Erasmus Medical Center. Offspring zygosity was identified using a copy-number duplex qPCR performed on phenol/chloroform/isoamyl-alcohol (Sigma Aldrich, St. Louis, MO, USA) extracted genomic mouse DNA from toe snip. TaqMan Genotyping Master

Mix (Life technologies, Carlsbad, CA, USA) with TaqMan uPA genotyping assay (Mm00422051_cn, Life Technologies) and Tert gene references mix (Life technologies) were used according to the manufacturer's protocol. All animal work was conducted according to relevant Dutch national guidelines. The study protocol was approved by the animal ethics committee of the Erasmus Medical Center (DEC nr 141-12-11).

Human hepatocyte transplantation

uPA homozygous mice, 6-12 weeks of age, were transplanted as previously described [186]. In short, mice were anesthetized and transplanted via intrasplenic injection with 0.5-2 x 10^6 viable commercially available cryopreserved human hepatocytes from a single donor (Lonza, Basel, Switzerland). Graft take was determined by human albumin in mouse serum using enzyme-linked immunosorbent assay (ELISA) with human albumin cross-adsorbed antibody (Bethyl laboratories, Montgomery, Tx, USA) as previously described [186].

Mouse infection

Mice were inoculated intravenously (i.v.) with 135-200 µl pooled patient EDTA-plasma (6.8 log international units (IU)/ml), individual patient EDTA-plasma (6.7 log IU/ml), homogenized liver biopsy fragment (6.3 log IU/ml), feces (8.8 log IU/ml or diluted to 6.8 log IU/ml) or culture supernatant from a P7 of feces (7.4 log IU/ml) containing HEV gt 3 as denoted in Table 1. This study was approved by the medical ethical committees of Erasmus MC and Antwerp University Hospital.

Histology, immunohistochemistry and HEV ORF2 immunofluorescence

To confirm *in vitro* HEV replication after 7 to 14 days post infection, cells were fixed in 80% acetone (Sigma-Aldrich) for 10 minutes, washed 3 times with PBS and air-dried. Cells were then blocked for 30 min at 36.5°C with 10% normal goat serum (MP Biomedicals, Santa Ana, CA, USA), followed by three times washing in PBS. Subsequently cells were stained for 1 hour at 36.5°C with a 1:200 0.5% BSA/PBS diluted goat-α-HEV open reading frame (ORF) 2 aa434-547 antibody (MAB8002, Merck-Millipore, Billerica, Massachusetts, USA), followed by staining with 1:200 0.5% BSA/PBS diluted goat anti mouse IgG conjugated with Alexa Fluor 488 (Life Technologies) for 1 hour at 37°C. After washing with PBS, cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and pictures were taken using a confocal microscopy (ZEISS LSM700).

Mouse livers were fixed in 4% formaldehyde solution (Merck-Millipore). Standard H&E staining was performed and human hepatocytes were identified using goat

anti-human albumin cross-adsorbed antibody (Bethyl laboratories) or mouse anti-human mitochondria antibody (Merck). To visualize the detected antigens, 3,3'-diaminobenzidine (DAB, DAKO, Copenhagen, Denmark) was added as a substrate and slides were counterstained with hematoxylin.

HEV RNA detection

All samples were screened for the presence of HEV RNA by an internally controlled quantitative real-time RT-PCR, described previously [90]. The qPCR had a lower limit of detection in EDTA-plasma (95% hit rate) of 143 IU/ml as determined by the 1st World Health Organization standard for HEV RNA NAT-Based assays (6329/10, Paul Ehrlich Institute, Germany). Ct values above 38 were considered background and given the arbitrary value of 0. HEV RNA detection in serum samples with Ct values below 38 are indicated with their calculated values. Feces was pre-treated with transport and recovery buffer (STAR buffer, Roche, Almere, The Netherlands) and chloroform. Liver tissues were homogenized using ceramic beads in 500 µl RPMI (Lonza). Mouse serum, bile and liver homogenate supernatants, were diluted 10x before extraction due to limited sample volume or to dilute any impurities inhibiting the qPCR.

Statistics

Graphpad Prism 5.01 was used for statistical analysis. Michaelis-Menten non-linear test was used to determine goodness of fit and Kruskal-Wallis one-way Anova test was used to calculate the p-value. Significance was set at $P<0.05$.

RESULTS

HEV gt 3 from different clinical sources efficiently infects A549 cells in vitro

HEV gt 3 derived from patient HEV0069 feces was able to infect cultured A549 cells with HEV RNA titers up to 7 log IU/ml in culture supernatant within 13 days post infection (p.i.). HEV was passaged seven times onto new cells, which resulted in increasing HEV RNA titers after each passage up to 8 log IU/ml (Figure 1A). After establishment of efficient HEV infection in the A549 cell culture system, isolates from 8 chronic HEV patients were used to study the differences between infectivity of different specimen types (feces, EDTA-plasma and a liver biopsy) *in vitro*. Viral load was assessed weekly by qRT-PCR. Figure 1 clearly shows differences in infectivity after one passage on A549 cells. Feces- (striped lines) and liver-derived HEV (blue line) show more efficient propagation

than EDTA-plasma samples (green lines). This finding was confirmed *in vitro* by a generally accepted TCID50 assay. Culture supernatant of feces (HEV0069) after 1 passage and 7 passages had a 4.8 and 5.5 log TCID/ml respectively, whereas culture supernatant derived from EDTA-plasma (HEV0014) had only an infectivity of 2.5 log TCID/ml. Anti-HEV ORF2 fluorescence staining confirmed HEV protein expression in feces-inoculated A549 cells, but not in uninfected control cells (Figure 1B). HEV is visualized in cytoplasm of clustered infected cells without obvious cytopathogenic effects. The percentage of infected cells was estimated at about 10% of total cells.

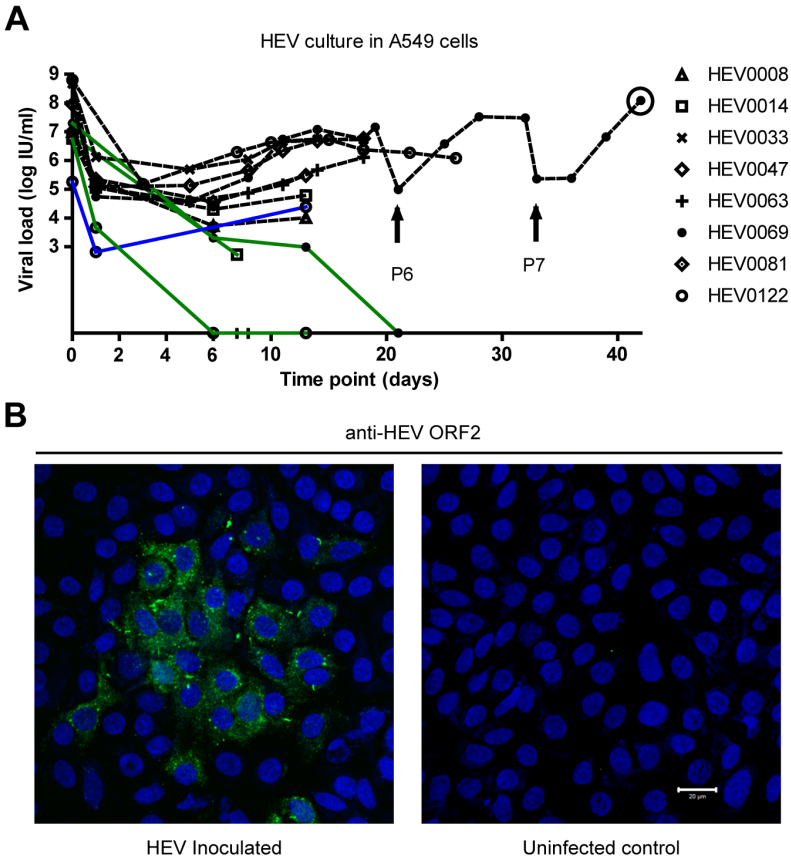


Figure 1 Differences in in vitro infectivity of HEV gt 3 containing clinical samples on A549 cells. A) Stable in vitro HEV replication of feces from multiple patients and liver in A549 cells (striped and blue lines respectively) and less efficient propagation of HEV-antibody free serum (green lines). HEV RNA was quantified with qRT-PCR in supernatant. The concentration of the initial inocula is indicated on the Y-axis on time point 0. Arrows indicate first viral load quantification after new passage (P6 and P7). The circle around the last P7 viral load of patient HEV0069, indicates the inoculum used for in vivo challenge. B) HEV ORF2 immunofluorescence of HEV infected and uninfected control A549 cells.

Successful infection of chimeric mice with feces-derived HEV gt 3

uPA-NOG mice, successfully transplanted with human hepatocytes (see Methods) had increasing human albumin levels in serum (Figure 2A), which correlated with liver repopulation by human hepatocytes as demonstrated by H&E; specific human albumin and human mitochondria staining (Figure 2B) [186]. To corroborate the observed feces-derived HEV *in vitro* infectivity, filtered undiluted fecal suspensions (see Methods) from patients HEV0069 and HEV0122 were i.v. inoculated into chimeric mice and the infection course was documented for 2, 6 or 14 weeks until sacrifice. During follow-up, HEV RNA was detected in feces of these mice with titers up to 7 log IU/gram (Figure 2C), while HEV viremia was low and inconsistently detectable, with maximum viral loads of 3.6 log IU/ml (Figure 2D). All 10 inoculated animals had high intrahepatic HEV RNA titers at sacrifice (up to 8 log IU HEV RNA/gram, Figure 2E), confirming the *in vivo* infectivity of feces-derived HEV gt 3 with establishment of 100% persistent infections.

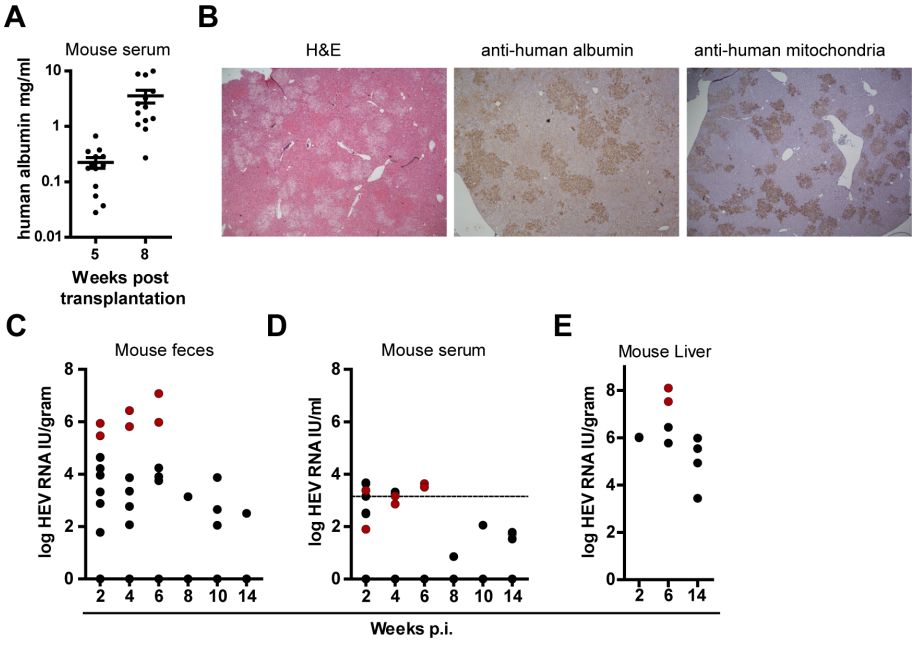


Figure 20 Successful infection of human liver-chimeric mice with HEV gt 3 derived from feces from two chronic HEV patients. A) Human albumin levels were measured in mouse serum via ELISA to quantify the hepatocyte graft take at 5 and 8 weeks post transplantation (n=13, mean ± SEM). B) Liver histology at 8 weeks post transplantation; H&E (left panel), anti-human albumin (middle panel), and anti-human mitochondria staining (right panel). Chimeric mice (n=10) were challenged i.v. with 8 log IU HEV RNA derived from human feces from patient HEV0069 (black dots) or patient HEV0122 (red dots) for 2, 6 or 14 weeks (n=2; 4; 4 respectively) (C-E). HEV RNA levels were measured by qPCR in mouse feces (C), mouse serum (D), and in mouse liver (E). Lower limit of detection in serum is 3.2 log IU/ml (dashed line; D).

Differences in in vitro HEV gt 3 infectivity are reflected in vivo

In order to assess the infectivity differences of HEV RNA containing clinical samples a total of 19 chimeric mice were challenged with EDTA-plasma samples from patients HEV0014 and HEV0122, with liver homogenate from patient HEV0122, with feces of patient HEV0069 or with a high titre P7 culture supernatant of this patient's feces (Table 1, Figure 3A). Care was taken to inject animals with similar HEV RNA containing inocula, with some variation due to differences in injected volumes (135 to 200µL). The respective inocula are indicated below the X-axis (Figure 3A). Given the need for liver HEV RNA determination to demonstrate or rule out HEV infectivity, large liver fragments of sacrificed animals were collected at week 6 to week 17 after inoculation. Only liver- and feces-derived inocula proved to be infectious in chimeric mice. Serum or A549 cell culture-derived inocula did not result in detectable HEV RNA levels at 6, 14 or 17 weeks after inoculation in any of the examined biological matrices (feces, sera, bile and liver; Figure 3A and data not shown). In addition, untransplanted $uPA^{+/-}$ mice inoculated with undiluted fecal suspensions from patient HEV0069 (8 log IU HEV RNA) remained HEV RNA negative in liver, serum and feces, indicating that human hepatocytes are HEV target cells *in vivo* (n=3; data not shown).

The absence of detectable HEV RNA could not be ascribed to loss of chimerism, as none of the HEV challenged human liver chimeric mice had a critical drop in human albumin during the course of the experiment (Figure 3B). Intrahepatic HEV RNA titers do vary and correlate with the degree of liver chimerism, as reflected by the human albumin levels in mouse serum (R^2 0.6824) (Figure 3C). However, the latter does not explain the infectivity differences observed between the different HEV containing samples, as the animal with lowest human albumin values at end of follow-up (50 µg/ml) still had detectable intrahepatic HEV RNA levels. These data therefore corroborate a genuine biological difference in infectivity of HEV containing samples of different origins.

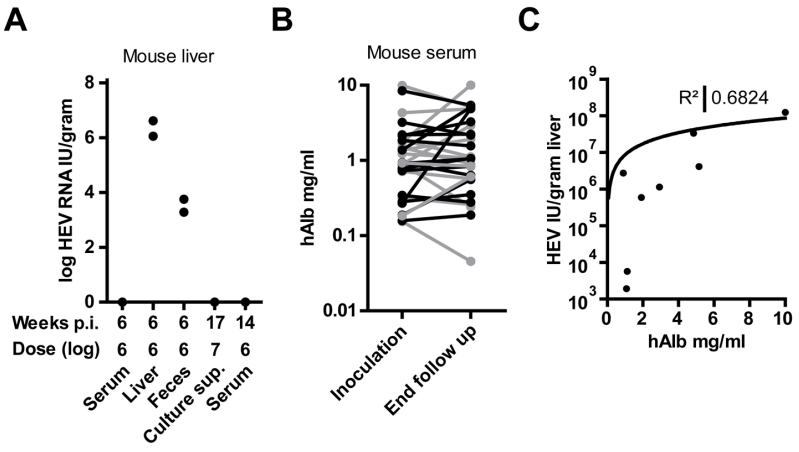


Figure 3 Differences in in vivo infectivity of HEV gt 3 containing clinical samples in human liver chimeric mice. Chimeric mice (n=19) were challenged i.v. with HEV RNA containing inocula derived from EDTA plasma (patient HEV0014 and HEV0122; n=4 respectively), a cryopreserved liver biopsy (patient HEV0122; n=2), feces (patient HEV0069; n=2), or P7 culture supernatant of patient HEV0069 feces (n=7) (see Methods). The respective infectious doses are indicated below the X-axis, as well as the duration of the infection in weeks. HEV RNA levels were measured by qPCR in mouse liver at sacrifice (A). B) Serum human albumin levels at HEV inoculation and at end of follow up of HEV RNA negative (Black lines) and HEV RNA positive (Gray lines) mice (n=29). C) Non-linear regression of chimerism, indicated by human albumin level in mouse serum (X-axis), versus the HEV titer in the liver (Y-axis) at week 6 post infection.

HEV viral shedding follows the natural human route in infected chimeric mice

HEV is preferentially secreted via feces in infected individuals. In fact, HEV seroconverted patients continue to shed HEV in feces for several weeks, even when HEV serum titers drop to undetectable levels [182]. We found that in infected animals serum titers were lower compared to feces and liver titers. In 6 infected mice, the average HEV RNA titers in serum, feces and liver were 2.9±0.4 log IU/ml, 4.9±0.7 log IU/gram, 6.3±0.4 log IU/gram, respectively (mean ± SEM; P-value 0.005 for difference, Kruskall-Wallis test). In addition, in animals from which bile could be obtained, average bile HEV RNA titers (6.4 log IU/ml) were in the range of those of positive feces samples (6.2 log IU/gram), demonstrating that virions are secreted preferentially through the biliary canaliculi, instead of basolaterally in the liver sinusoids.

Although serum levels are low during the course of the infection, HEV RNA was detected in liver of all HEV inoculated chimeric animals once HEV RNA became detectable in feces, demonstrating the 100% chronicity rate upon established infection. Thus, in live animals, feces, rather than serum, is a specific read-out for infection success.

DISCUSSION

Human HEV gt 3 is reported to be infectious for several non-human primates, rabbits and pigs [183]. However, none of these potential animal models for the study of HEV develop a chronic infection, nor are there overt hepatitis signs. In this study we applied a human-liver chimeric mouse model and A549 cell culture system to establish and examine the infectivity of HEV gt 3 containing clinical samples. We demonstrated that *in vitro* propagation of feces- and liver-derived virus isolates is higher than that of EDTA-plasma derived inocula and that these infectivity differences *in vitro* are corroborated *in vivo* in chimeric mice.

Our data show that human liver-chimeric mice can develop a 100% chronic HEV infection, mimicking the infection course in solid organ transplant and bone marrow transplant recipients. In addition, HEV in these mice is preferentially shed in bile and feces, which corresponds to the viral pathobiology seen in humans. This chimeric small animal model may therefore fill an important gap in preclinical antiviral efficacy studies.

Feces- and liver-derived inocula induced gradual increases of HEV RNA levels in the A549 cell culture system from day 6 onwards. HEV cell surface detachment and gradual release into the supernatant at the first day after inoculation may have caused the relative high viral titers early on, which decreased further for plasma inocula and plateaued for both liver-and feces-derived viruses. We observed intrinsic HEV infectivity differences that were most apparent when isolates from plasma, feces and liver obtained from a single patient (HEV0122) were examined: only the latter 2 resulted in prolonged replication, both *in vitro* and *in vivo*. HEV virions from plasma and feces have been found to differ in virion density, ascribed to a divergent lipid membrane content on their surface. In addition, culture derived HEV has comparable characteristics to plasma derived HEV [187, 188]. Differences between these viruses are probably caused by the detergents and proteases secreted by the pancreas, which strips the HEV virions from their lipid membrane upon their passage in the intestinal system [189]. This different buoyant density may influence *in vivo* infectivity, as has previously been shown for hepatitis C virus [190]. Circulating inhibiting factors, including virus-specific antibodies, on the other hand may also negatively influence the infectivity of the virus preparations. However, we selected only pre-seroconversion plasma samples for *in vitro* and *in vivo* infectivity assays, but could not obtain productive infections.

In contrast, one group demonstrated efficient *in vitro* HEV replication from serum samples of acute HEV gt 3 patients irrespective of coexisting HEV antibodies with

rapid viral replication from day 2 post-inoculation onwards [191]. Others showed that chronic HEV gt 3 sera, but not acute phase sera, yielded productive infections with insertions in the ORF1 region after 5 to 6 weeks of culture [192-195].

The observation that human plasma-derived virus is less infectious *in vivo* may be relevant for the infectivity and epidemiology of HEV in humans. Indeed, only a limited number of cases of transfusion transmitted HEV have been reported despite administration of contaminated blood products [175, 182, 196, 197]. In addition, a recent retrospective survey of United Kingdom's plasma pool, surprisingly showed that only half of HEV viremic British blood donors infected their recipients [175]. The HEV transmission rate seemed to be dependent on the HEV RNA load and anti-HEV antibodies status in donor plasma. Using currently described tools, it will be interesting to examine the critical factors associated with these infectivity differences, including the role of different HEV genotypes, membrane lipid content and infectious dose. While recent reports have shown HEV gt 3 viremia among blood donors [178], Dutch national blood safety guidelines do not require NAT testing of the donor pool [198]. This is of concern for solid organ transplant patients who are prone to develop chronic infections, which may quickly progress to severe liver fibrosis.

In conclusion, we have shown that feces- and liver-derived HEV gt 3 induces a sustained infection in human liver-chimeric mice with preferential viral shedding towards mouse bile and feces, mimicking the course of infection in humans. Besides antiviral efficacy studies, this novel small animal model offers new avenues to study HEV pathogenesis.

ACKNOWLEDGEMENTS

We would like to thank Vincent Vaes from the Erasmus MC animal care facility for his assistance in performing biotechnical manipulations and Jolanda Voermans, Jolanda Maaskant, Kim Kreeft, Marwa Karim and Stalin Raj for excellent technical assistance.

Chapter 5

Summarizing discussion

Taken in part from

Hepatitis E virus infection in hematopoietic stem cell transplant recipients

Annemiek A. van der Eijk, **Suzan D. Pas**, Jan J. Cornelissen, Robert A. de Man

Current Opinion Infectious Diseases 2014, 27:309–315

Guillain-Barré syndrome associated with preceding hepatitis E virus infection

Bianca van den Berg, Annemiek A. van der Eijk, **Suzan D. Pas**, Jeremy G. Hunter, Richie G. Madden, Anne P. Tio-Gillen, Harry R. Dalton, D., Bart C. Jacobs

Neurology, 2014;82:491–497

The main goal of this thesis was to study the disease burden of HEV infections in cohorts of immune-compromised patients. To this end we optimized diagnostic assays and studied HEV pathogenesis in patients and in an *in vivo* model for HEV infection.

HEV DIAGNOSTIC ASSAYS

Until recently, testing of immune-compromised patients for HEV infections was mostly performed - if at all - with assays detecting HEV specific antibodies but lacking sensitivity and specificity, like antibody ELISAs and Western blots. From our studies, in which we compared frequently used ELISA systems, it became clear that only a minority of the available assays tested was capable to sensitively and specifically detect HEV antibodies. We showed, that in immune-compromised patients (solid organ and hematological transplant recipients) anti-HEV IgM and anti-HEV IgG antibody responses measured by ELISAs, were detected later with a median of 64 and 129 days respectively, as compared to first HEV RNA detection. Therefore we recommended to test immune-compromised patients, rather with sensitive molecular techniques, like real time RT-PCR, than with serological techniques. However in immune-competent individuals presenting with acute hepatitis, ELISA techniques may be used, since they can differentiate between recent (IgM) infections and those of the more distant past (IgG). This can be done at lower cost than HEV RNA detection, which decreases the diagnostic barrier. However, one should bear in mind that re-infections with HEV may occur, with HEV IgG antibody and RNA being simultaneously present in peripheral blood. Other techniques like, HEV antigen detection may also be used in the absence of HEV RNA testing. However the so-far only commercially available antigen detection assay has been reported to have a sensitivity of approximately 60% and specificity of approximately 86% as compared to RT-PCR[199]. *In vitro* HEV isolation, which has recently been developed for research purposes, cannot be considered a diagnostic tool, because of its relatively long turnaround time and low sensitivity as compared to molecular and serological assays.

CLINICAL ASPECTS OF HEV INFECTION

Acute hepatitis E in developed countries

Zoonotic autochthonous hepatitis E infections have been identified in Japan (gts 3 and 4) Europe, New Zealand, and to a lesser extent in the USA[159]. The majority of hepatitis E cases in Europe are caused by HEV gt 3. Patients infected with HEV gt 3 often remain asymptomatic. Only 2-33% of infected individuals present with usually non-specific symptoms and signs like abdominal pain, malaise, fever, although some present with jaundice.[152] A recent study has documented HEV gt 4 in European pigs. Two human HEV gt 4 clusters have been documented in France and Italy[200-202] and two apparently isolated cases in Denmark and The Netherlands (unpublished data, Pas *et al*). Infections with HEV gts 3 and 4 cause rather similar clinical manifestations. The acute hepatitis associated with gt 4 infection tends to be more fulminant[203]. Excess mortality during pregnancy is not seen in association with infection by either of these genotypes. In developed countries HEV infection appears to cause symptomatic hepatitis more commonly in middle-aged and elderly men (M:F ratio 3:1). The reason for this skewed distribution is not clear, but may be explained by a higher prevalence of pre-existing subclinical hepatic fibrosis in such individuals[204, 205]. It is interesting to note that in patients treated with immunosuppressive drugs for inflammatory arthritis, acute hepatitis E has been recently described. All HEV infected patients in that study had a favorable outcome after three months[206].

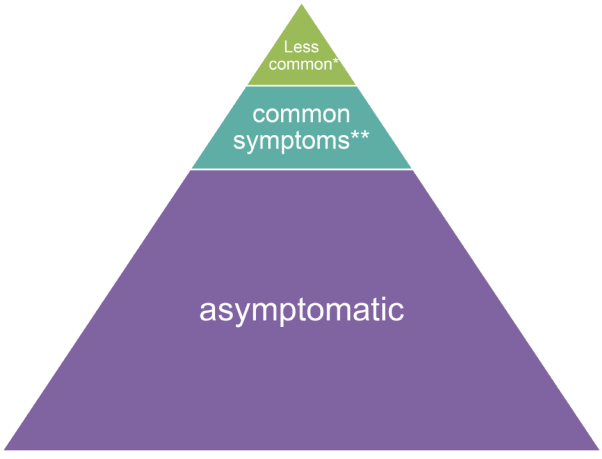


Figure 1 Acute HEV gt 3 symptoms. The majority of infected individuals is asymptomatic, a relatively small proportion had common symptoms, like jaundice, anorexia, lethargy, abdominal pain and vomiting (**). A minority of HEV infections shows signs of myalgia, pruritus, weight loss, headaches, arthralgia and neurological signs(*).

With the exception of HEV-associated neurological syndromes (see below: Extrahepatic manifestations), the symptoms and signs of hepatitis E are rather non-specific and indistinguishable from other causes of viral hepatitis (Figure 1). Most patients recover within 4-6 weeks, although in patients with pre-existing liver disease the prognosis is poor due to hepatic failure[16].

Chronic hepatitis E

Chronic HEV infection caused by HEV gt 3 is defined by the presence of HEV RNA in plasma and/or stool detected by RT-PCR for 6 months or more[69]. To date no chronic hepatitis has been reported with genotypes 1, 2 or 4.

We and others have reported chronic HEV infection in immune-compromised patients, including solid organ transplant recipients, recipients of allogeneic stem cell transplantation (alloHSCT) and HIV-infected patients [69, 90, 107, 156, 207]. It has not been found in patients with inflammatory arthritis treated with immune-suppressive drugs, who do develop acute hepatitis E [206]. A prevalence of 1-3% of hepatitis E viremia in recipients of solid organ transplants has been reported, with 47-83% of the patients developing chronic hepatitis[69, 90, 112]. Most immune-compromised patients have no clinical symptoms at presentation, but usually show increased transaminase levels [71, 89, 156]. In a recent study, the clinical course of HEV infection in 34 immune-compromised patients in The Netherlands was described. Median ALT levels in patients with a chronic infection were significantly lower (106 U/l) compared to the levels found in patients who had spontaneous viral clearance within 6 months (1087 U/l) ($p=0.002$)[208]. Similar findings were reported from a larger cohort of mainly European transplant recipients[71].

Several case reports have described chronic HEV infections in HIV infected patients with $CD4^+$ counts lower than 250 cells/μl [207, 209]. More recent studies suggest that HIV-infected patients are not at increased risk of acquiring HEV infection, but are at a higher risk of developing chronic hepatitis E.

Screening of two cohorts of 72 and 52 alloHSCT patients respectively, failed to document cases of HEV infection or reactivation, which did not suggest in increased risk for such patients [135, 137]. In contrast, our more extensive retrospective cross-sectional study in a cohort of 328 alloHSCT patients showed an incidence of 2.4% HEV infection[156]. Our study had a median follow-up time of 41 months and chronic hepatitis followed the acute infection in five out of eight acute HEV cases (63%). In the HEV infected patients, liver enzyme abnormalities had been considered to be related to hepatic GVHD in five (63%) patients, and drug induced liver injury in three (38%) patients. One patient was diagnosed with HEV reactivation after a preceding infection prior to alloHSCT. This is the second

case of HEV reactivation after alloHSCT described in literature so far [136, 156]. Diagnosis of HEV infection in these patients is hampered by relatively low peak aminotransferase levels compared to non-immune-compromised patients[68], which may be explained by intensive immune-suppressive therapy suppressing inflammation.
In recipients of solid organ transplants the use of tacrolimus rather than cyclosporine A as a main immunosuppressant and a low platelet count at diagnosis of HEV infection are two independent variables that predict the development of chronic HEV infection[71]. Progression to chronic HEV in alloHSCT patients is less well characterized, but may be explained by impaired immune reconstitution, including insufficient lymphocyte count recovery, which are well known risk factors for post-transplantation infections[140-142]. In particular, impaired reconstitution of $CD4^+$ and $CD8^+$ T-cells predisposes for higher morbidity associated with infectious diseases in these patients[143].

Rapid progression of fibrosis and cirrhosis in chronically infected HEV transplant recipients has been described[122]. Liver histopathology in chronic HEV infected heart transplant recipients for instance showed advanced fibrosis within 2 years after infection[89]. Biopsies of patients with chronic HEV showed typical signs of active viral hepatitis with inflammatory activity, councilman bodies and acidophilic degeneration[89, 156]. However, no distinct pathognomonic features differentiating HEV infections from hepatitis B or C have been identified.

HEV induced acute on chronic liver failure

The definition of acute-on-chronic liver failure (ACLF) according to the European society of liver diseases is 'acute deterioration of pre-existing, chronic liver disease, usually related to a precipitating event and associated with increased mortality at 3 months due to multisystem organ failure [210]. These precipitating events are for instance DILI, alcoholic hepatitis, viral hepatitis preceding the ACLF, but also events like trauma and variceal bleeding. In the event of a HEV superinfection, the liver quickly deteriorates, leading to a multi-organ failure and in some cases death. Several studies in HEV gt 1 endemic countries reported a median 21% (range, 4-72%) of the cases with HEV as cause of the ACLF[211]. However, in China, endemic for HEV gt 3 and 4; a large study of HEV vs HAV induced ACLF in chronic hepatitis B carriers showed that outcome of HEV super-infections is generally more severe than those of HAV. Significantly more patients had complications (94.9 vs 61.5% respectively), hepatic failure (39.7 vs. 11.5% resp.) and died (33.8 vs 1.9% resp.). When looking at the laboratory results, the

peak levels of serum bilirubin, serum albumin and pro-thrombin activity were significantly higher in the HEV induced ACLF group than in HAV induced ACLF patients.
The vast majority of studies on HEV induced ACLF have been performed in Asia or Africa, the literature is very limited on HEV induced ACLF in Europe. According to Jalan *et al*. the main causes of ACLF in Europe are bacterial infections and alcoholism, but since HEV awareness is just rising and diagnostic methods were not accurate until recently,, the incidence of HEV induced ACLF still needs to be studied in Europe[212]. One study by Peron *et al*, revealed a staggering 71% mortality rate in patients with underlying hepatic disease (either alcoholism or chronic liver disease) who had a fulminant hepatitis due to a HEV infection.[154]
In Erasmus MC we have seen 3 patients with pre-existent liver diseases as underlying morbidity, dying shortly after HEV induced ACLF associated with liver related morbidity[213], confirming that this serious condition due to HEV induced ACLF may also occur in Europe.

Extrahepatic manifestations
There have been a number of case reports associating HEV infection with extra-hepatic manifestations, including pancreatitis, acute thrombocytopenia, aplastic anaemia, and renal disease[16, 214]. In addition, 5% of HEV cases present with a neurological manifestation[215]. At first, scarce case reports suggested that HEV may be responsible for atypical neurological symptoms. HEV RNA was detected in the serum and cerebrospinal fluid in a kidney-transplant patient who was chronically infected by HEV. The patient showed symptoms of peripheral nerve involvement with proximal muscular weakness that affected the four limbs joints with central nervous-system involvement and bilateral pyramidal syndrome[216].
HEV-associated neurological injury has been documented in both acute and chronic HEV infection, and in some cases HEV has been found in the cerebrospinal fluid[217]. In general, HEV has been associated with a palette of neurological manifestations, such as Bell's palsy, Guillan Barré syndrome (GBS) and neuralgic amyotrophy[218-220]. Case reports on GBS and HEV come from both developed and developing countries, suggesting that this condition is not genotype specific. The majority of GBS patients report respiratory or gastrointestinal tract infection before the onset of GBS. Infectious agents like *Campylobacter jejuni,* cytomegalovirus (CMV), Epstein-Barr virus (EBV) and *Mycoplasma pneumoniae* have been associated with GBS[218]. However, in our case-control cohort study, we showed that 10 (5%) of 201 Guillain-Barre syndrome patients from The Netherlands had HEV infection at the start of their illness[218]. The clinical features

and outcome in these patients were similar to those found in Guillain-Barre syndrome cases not associated with HEV. The pathophysiological mechanisms of HEV-associated neurological injury remain unclear. The role of HEV infection in the pathogenesis of GBS most likely falls within the existing paradigm of a post infectious immune-mediated complication, rather than being caused by the infection itself. The presence of anti-ganglioside GM2 antibodies upon HEV infection was associated with GBS, suggesting molecular mimicry involving gangliosides[221]. We showed that the incidence of recent HEV infections -based on the presence of anti-HEV IgM- was ten times higher than in a similar group of healthy controls. The majority of HEV-associated cases of GBS in this large cohort had a sensory-motor and demyelinating form of GBS, the predominant subtype of GBS in the Netherlands. In all 10 cases of HEV-associated GBS, the neurologic illness completely dominated the clinical picture: all were anicteric, and the alanine aminotransferase was less than 150 IU/L in 9 of these cases.

A further study of UK and Dutch patients with brachial neuritis showed that 5 of 47 (10%) of cases had an associated acute HEV gt 3 infection at the start of their neurological illness[219]. All the HEV-associated NA cases had bilateral involvement of the brachial plexus, compared to 15/35 (43%) of the Dutch cohort without recent evidence of HEV infection. HEV-associated NA patients were aged between 34-40 year. All patients were anicteric with only mildly raised ALT's (in some cases the ALT was normal), and some were viremia at presentation. Anti-HEV IgM positivity was not related to age, gender, disease severity or outcome in these patients

The latter observation raises the question whether early anti-viral therapy may improve the natural history of either condition. This may be facilitated by either cito-diagnostic testing or the use of a (bedside) HEV antigen detection assay, described above (chapter 5.1).

The pathophysiological mechanisms of HEV-associated neurological injury are unknown and need to be studied in more detail. Especially when neurological features dominate the clinical presentation, the diagnosis can easily be missed.

HEPATITIS E VIRUS IN BLOOD AND BLOOD PRODUCTS

Antibodies directed against HEV and HEV RNA have been found in donated blood in a number of countries (Table 2). Asymptomatic infection is common and accounts for the large numbers of blood donors who are viraemic at the time of donation. The majority of blood products is administered to the most vulnerable patient group, the immune-compromised, who are at increased risk for developing chronic HEV, with all clinical consequences described in this thesis.

Due to the use of relatively insensitive serological assays, sero-prevalence has been underestimated in the past. Table 2 shows an overview of published studies in Europe, China and the USA (mainly HEV gt 3 endemic countries) in which sero-prevalence and HEV viremia in blood donors were assessed. In some studies the same dataset was assessed with different assays showing up to seven times underestimation of the actual sero-prevalence when measured with insensitive ELISAs[96]. With the increased performance of the anti-HEV IgG ELISAs, it has become clear that the sero-prevalence is higher than previously assumed, ranging from 3.4% in Scottish blood donors to up to 52% in the south-east of France (Table 2). These data suggest that HEV is more endemic in south-east France, and these high sero-prevalence rates are in accordance with high numbers of blood donors who are viraemic at the time of donation (Table 2).

Multiple well-designed studies in the Dutch, American, British and Danish blood donors showed a high correlation between age and HEV antibody sero-prevalence[176, 222-224]. It has been demonstrated that when evaluating sero-prevalence levels, indeed we face an age cohort-effect. This means that not only as people get older they have encountered more HEV infections, but also that the prevalence of HEV infection in the population was higher in previous decades. This resulted in a relatively high sero-prevalence today due to the detection of long persisting HEV antibodies in the elderly.[176]

Table 2 HEV viraemia and seroprevalence in blood donors

Country	Year of sampling	Blood donors HEV RNA +	HEV IgG sero-prev.	Assay	Reference
SW France	2003-2004	-	16%*	Adaltis	Mansuy et al, 2008 225
	2003-2004	-	52.5%*	Wantai	Mansuy et al, 2011 60
	2011	-	39.1%*	Wantai	Mansuy et al, 2015 226
Germany	1996	-	50.7%*	Wantai	Wenzel et al, 2014 177
	2010	-	29.5%*	Wantai	Wenzel et al, 2013 96
	2010	-	18.0%*	Mikrogen	Wenzel et al, 2013 96
	2010	-	4.5%*	MP diagnostics	Wenzel et al, 2013 96
	2011	1:1200	-		Vollmer et al, 2012 227
	-	1:4525	-		Baylis et al, 2012 146
	2011	-	34.3%	Wantai	Wenzel et al, 2014 177
Netherlands		-	1.1%*	Abbott	Zaaijer et al, 1993 228
	1988	-	46.6%	Wantai	Hogema et al, 2014 176
	2000	-	27.3%	Wantai	Hogema et al, 2014 176
	2011	1:2671	27.0%	Wantai	Slot et al, 2013 222
	2013	1:1761	-		Zaaijer, 2014 229
	2014	1: 611	-		Zaaijer, 2014 229
England	-	-	3.9%*	Abbott	Bernal et al, 1996 230
	1991	-	13.0%	Wantai	Ijaz et al, 2009 223
	2004	-	13.5%	Wantai	Ijaz et al, 2009 223
	2007	1:7000	-		Ijaz et al, 2012 231
			10.0%*	Wantai	Beale et al, 2011 65
	2012-2013	1:2848	-		Hewitt et al, 2014 175
Sweden			9.2%*	Abbott	Olsen et al, 2006 232
		1:7986	-		Baylis et al, 2012146
N-Austria		1:8416	13.5%	Wantai	Fischer et al, 2015 233
Scotland		1:14.520	4.7%*	Wantai	Cleland et al, 2013 234
Spain	2013	1:3333	10.7%	Mikrogen	Sauleda et al, 2015 235
	2013		20.0%	Wantai	Sauleda et al, 2015 235
China	2005-2006	-	23.5%*	MP diagnostics	Jia et al, 2015 236
	2002-2008	-	32.6%	Wantai	Guo et al, 2010 237
USA		Nil	-		Baylis et al, 2012 146
		Nil	18.8%	Wantai	Xu et al, 2012 224

The above data are from blood donors with the exception of those marked * which are from 'healthy adults'. Assays in italics have a relatively low sensitivity41, 238.

Studying longitudinal samples from 23 donors collected over 22 year-period, it was shown that the Wantai assay consistently detected long persisting HEV specific antibodies. Furthermore, data from The Netherlands (Table 2) and Germany, suggest that HEV infections have come in waves during the past decades: by the end of the 1980's there was a high prevalence of roughly half of the German and Dutch populations had been infected with HEV, whereas the sero-prevalence had declined towards 27-29% by the end of 2010's. An increase of HEV infections was already detected in the Netherlands from 2011 onwards, although this coincided with increased awareness and sensitivity of diagnostic assays. More recent data from the Dutch blood donors show a continuing upward trend, with currently 1:611 donors being HEV viremic[229]. Since HEV is not a notifiable disease in the Netherlands, there are no well-defined national data to support this finding. However, the Dutch National Institute of Public Health and the Environment (RIVM) does acknowledge an increase in the incidence of HEV infections from 2014 onward, based on voluntary notification of Dutch diagnostic laboratories (Adviesbrief deskundigenberaad DB-Z bij hepatitis E, dd 26-06-2015), which cannot be explained by an increase in sensitivity of diagnostic assays alone. Other evidence for the increase in the incidence of HEV infections in The Netherlands is an overall increasing sero-prevalence (19.8% in 1988, 7.0% in 1995, 4% in 2000 and 12.7% in 2011) in the last 10 years among young adults (18-21 years). Prevalence studies among this age category over time suffer to a much lesser extent from an age-cohort bias.

Several reports have shown that HEV can be transmitted via blood transfusion[150, 151, 196, 197, 239], though specific risk factors for transmission have poorly been defined. Transmission via blood products may well be less efficient than feco-oral- transmission, as blood products originating from more than one donor, may contain HEV neutralizing antibodies that may interfere with transmission. The first blood donor transmission study was conducted in the UK, and included 225.000 donors of whom 0.04% or 1:2848 were HEV viremic. This study revealed HEV transmission in 18 of 43 (43%) exposed and followed up patients. Transmission proved to be dependent on factors like higher viral load, presence of donor HEV antibodies and recipients immune suppression. The viral load was on average 1.5 log higher (max up to 6 log IU/ml) and antibody titers were lower in the donations that transmitted HEV than those that did not. However in two single cases high viral loads (>5log) did not lead to transmission, but the authors did not specify if this could be attributed to the fact that this was a seropositive recipient or multiple donations were given, which had included sero-positive individuals[175].

Clinical consequences of transfusion-transmitted HEV are still largely unknown. The sole transmission study up to now was conducted in the UK and describes clinical consequences. However, this study is clearly biased, since the patients were actively followed up after confirmed HEV contamination of the donation. By doing this, they stopped the more severe clinical sequelae in infected patients who were treated for their hepatitis. Long term follow up without intervention, as the current situation is in non-screening countries, has not been reported, which could reveal worse clinical outcome. The authors describe that in the UK an estimate 1200 HEV containing donations are transfused per year, and taken the study results into account, 43% transmission and 2/3 of the infused patients clear the virus without medical intervention, resulting in roughly 180 patients each year that do need medical intervention due to the acquired HEV infection. As stated above, this would mean either tapering immunosuppressive drugs, ribavirin treatment or, in case of HEV-ACLF very costly intensive care admissions or even a liver transplantation.

INTERVENTION STRATEGIES

Currently, no preventive intervention strategies other than vaccination in China are in place world-wide,. Intervention strategies for hepatitis E can be conducted at different levels. The simplest intervention technique, at least to gain insight in the number of actual hepatitis E cases, is to make it a notifiable disease. In some countries, like Germany, this is currently done, but not in the Netherlands. The Dutch government now relies on voluntarily reported and therefore inaccurate data from diagnostic laboratories.

In terms food-safety, intervention strategies at the source and the end of this HEV transmission chain, may have a major impact. Since currently HEV is present in raw pig products[52, 240], these could be banned from consumption, or at least carry warnings as is also practised for raw cheese products to prevent *Listeria* infections. At the source, vaccination of fattening pigs could be a management measure aiming at preventing zoonotic transmission.. Although this is an enormous effort, with >12 million pigs in the Netherlands alone (source: CBS[241]), it could be a first step towards HEV-free swine products.

On an individual level, intervention can take place either before HEV infection – HEV vaccination, HEV-specific dietary advice to risk groups and screening of blood donations (discussed below) – or after HEV infection, by therapeutic management of HEV infections through lowering iatrogenic immune suppression and/or anti-viral therapy.

Treatment

Acute HEV

Currently there are no WHO nor EASL / AASLD guidelines for treatment of hepatitis E. Most cases of acute hepatitis E infection are self-limiting, requiring only symptomatic or no treatment. However a minority of the patients develop fulminant hepatitis, some of which may eventually require liver transplantation[72, 73]. Especially patients with severe hepatitis and underlying chronic liver disease have a poor prognosis, although currently several have been treated successfully with ribavirin[74].

Chronic HEV

Treatment of chronic HEV infection in transplantation patients is basically based on two strategies, reduction of immune-suppressive therapy and treatment with anti-viral agents. Peg-IFN-α-2a/b and oral ribavirin have been successfully used for treating chronic HEV infection in immune-compromised patients[89, 125, 127, 156, 164, 242, 243].

Reducing the dose of immune-suppressive drugs targeting T cells can lead to HEV clearance in up to one third of patients[76]. Other studies have shown more limited utility of reducing immune-suppressive therapy, as one study showed that only 2 out of 18 solid organ transplant recipients were able to achieve viral clearance with this approach[71, 76, 208]. A possible explanation is that the evolution of the HEV infection could be related to the type of solid organ transplant and the associated level of immune-suppression employed [208].

Peg-IFN-α-2a has been successfully used for treating HEV infection in liver- and kidney transplant recipients as well as in a HIV infected patient [39, 125, 127, 243]. Tapering immunosuppressive drugs or treatment with Peg-IFN-α-2a/b is not always possible or desirable due to the high risk of rejection, which may lead to chronic allograft dysfunction and death. For these patients treatment with ribavirin should be considered. Rapid clearance (within 3 months) of HEV RNA from plasma with normalizing ALT levels and sustained virological response are

observed in patients with chronic HEV infection after start of ribavirin treatment[77]. The optimal daily dose and treatment duration of ribavirin is unknown. In case reports and small case series sustained virological response has been described with daily dosages between 200 mg and 1200 mg[80]. Treatment durations of less than 3 months and dose reduction of ribavirin have been associated with viral relapses or breakthrough[242]. No viral relapse was observed following 5 months of ribavirin therapy for treatment of chronic HEV in 9 transplant recipients [77, 242]. An i*n vitro* study, using the HEV replicon system (chapter 4) has shown that a single mutation in RNA dependent RNA polymerase, found in a non-responding patient, was able to escape from ribavirin therapy by enhancing replication in human liver cells. The majority of ribavirin treated patients are sustained responders, but as the precise mechanistic effect of ribavirin treatment is not fully understood, further studies are needed to elucidate the mechanisms of this therapeutic intervention.

HEV screening

Blood donations in The Netherlands: 'to screen or not to screen, that is the question.'

Currently, blood donors are not routinely screened for HEV in The Netherlands. Whether they should be screened is subject of lively debate and on-going research. As stated above, if in the UK roughly 180 patients per year need medical care due to a donation-transmitted HEV infection, whilst in The Netherlands the percentage of HEV viremic donors is approximately 4.5 times higher than in the UK. Furthermore, the HEV sero-convertion rate, upon transfusion in the Netherlands is currently estimated at 1.1%. compared to the 0.1-0.2% in the Hewitt study. Therefore estimation of the number of Dutch recipients who become actively HEV infected is hard to compare with the UK situation, but the relative risk of becoming HEV infected by blood or blood products in The Netherlands may well be higher than in the UK, although the prevalence and levels of specific serum antibodies should also be taken into account in estimating this relative risk.[175, 176].

Currently, only HIV, HTLV-1, HBV, HCV and Syphilis (Lues) are infectious agents for which Dutch blood donors are routinely screened. In the last two decades (1995-2014) 381 regular repeated donors tested positive for one of these agents in the Netherlands (HIV-1 n=53, HBV n=110, HCV n=14, HTLV n=11 and Lues n=196) (personal communication Prof H. Zaaijer, Sanquin Blood supply, Amsterdam).

Compared to the other two agents causing viral hepatitis (HBV and HCV), the number of HEV viremic donors is much higher (1:611[229]).
It should however be realized that infectious HEV, in contrast to HBV and HCV, is virtually omnipresent, found at various places in the food chain, including in pork products, seafood, game but also in surface water. Although immune-compromised patients, who would be most at risk, do get dietary advise, this patient group may be expected to be exposed regularly to HEV infection. The question may be why to invest lots of money to get the blood chain HEV-free, and thereby losing valuable blood products. Therefore the second option, providing 'HEV-safe' blood products for risk groups like immune-compromised patients may be a better option.
Hepatitis E virus fits well into the group of other infectious agents (like B19 virus or CMV) for which currently selected safe blood products are produced. All these infections are highly prevalent, but only are a major problem for specific risk groups. The health council of The Netherlands has advised the minister of Health in 2002 to start providing 'B19-safe blood products', meaning presence of B19 antibodies in cellular products or B19 viral load $<10^4$ geq/ml in plasma[244]. However B19 antibody positive blood is considered 'safe' for cellular products, but for HEV it is not known to what extent the antibodies found in 27% of the Dutch blood donors can indeed neutralize infectious HEV. More HEV transfusion related infections were found when HEV antibody levels were low and viral loads were high[175]. This provides the basis for further studies to elucidate which antibody levels and which HEV viral loads could still be considered 'safe' for immune-compromised patients.
In evaluating the need for either systematic HEV donor screening, or developing a policy of 'HEV-safe blood' for risk groups, it is also important to consider the overall disease burden for patients receiving blood or blood products, who develop HEV infection. It has a broad range from no clinical symptoms at all, to death due to acute-on-chronic liver failure. It is also not yet clear what the disease burden is, expressed in disability-adjusted life years (DALY). Finally all measures considered should also be evaluated in the light of the overall HEV exposure rate due to 'natural' oral-fecal and zoonotic exposure.

Unfortunately all these considerations have not yet been included in cost-benefit or cost-effectiveness studies for providing 'HEV-safe' blood. Such studies are required to provide ammunition for further discussion and decision making on this topic.

Yearly transplantation screening

Given the disease burden of HEV infection on transplantation patients and the overall prevalence of HEV infection in The Netherlands, the question arises whether a policy of HEV screening of transplant donors and recipients before transplantation should not be adopted. In addition a subsequent annual HEV recipient screening, as also practiced for HAV, HBV and HCV should be considered As a direct result of prevalence studies in the Erasmus MC transplant recipient populations, the currently adopted algorithms for yearly transplant recipient screening have changed by adding HEV RNA for HTX and LTX. This will also be implemented for KTX recipients in the near future. Up until March of 2015 (~1.5 years of screening), 32 new HEV infected patients (40% of total HEV cases in Erasmus MC) were identified by this proactive screening program[213]. Regardless of a possible introduction of 'HEV–safe' blood products, this yearly Tx screening for HEV should be performed, as these patients also have a significant chance to be infected via other transmission routes than blood transfusion.

HEV vaccine

Although great efforts have been made to develop a HEV vaccine, there are still major knowledge gaps to be filled. First, due to the study designs and the limited follow-up time, it has yet to be determined what the protective mechanisms are or for short- and long-term protection against hepatitis E. This is the reason why, in 2014 a phase IV clinical trial was initiated, of which the data are not yet published. Also, subsequent safety and efficacy studies in specific patient groups like immune-compromised should be conducted, although currently available data seem to suggest that the vaccine is safe in accidentally vaccinated pregnant women and hepatitis B surface antigen positive (HBsAg+) patients, but these data need to be confirmed with larger numbers of participants.

The WHO recognizes that hepatitis E is a world-wide problem, and focuses its efforts on large outbreaks in low sanitized countries. Therefore a strategic advisory group of experts (SAGE) has been established in 2013, to write a WHO vaccine position paper on the use of hepatitis E vaccine, which was published in May 2015. In this position paper the WHO states that there is still insufficient data on the use of the current candidate vaccine in children <16 years and the cross-protection for genotypes 1-4, and therefore does not recommend the use of this vaccine in national vaccination programs at present[245], but acknowledge the benefits of use in outbreak situations.

MODELS FOR PATHOGENESIS AND TREATMENT EFFICACY STUDIES

Hepatitis E virus has been shown to replicate *in vitro* in A549 cells derived from human lung cancer and on PLC/PRF/5 cells – a human hepatocellular carcinoma cell line[189]. However, since the first publication a decade ago, to our best knowledge, there are currently only three groups (in Japan, Germany and the Netherlands) world-wide who have reported propagation of wild type HEV strains in vitro [189, 192, 246]. For research purposes, a one-cycle luciferase replicon system provided the tool to study basic elements of HEV replication in vivo and in vitro.[31, 247] Using this replicon system, in vitro evidence has been provided on the efficacy of ribavirin and α-interferon, which was supportive of clinical findings with off-label use of these antivirals.[248]. However, for pathogenicity studies, several animal models have been described, like non-human primates, swine and rabbits, each with their specific advantages and disadvantages[249]. Non-human primates are only used nowadays when there is more benefit to human beings and if there is no other animal model available. These animals do not develop major clinical signs, only one of four had elevated ALT levels. Swine are only used for gt 3 and 4 infections and only show moderate pathology. Rabbits, have been shown to be susceptible for HEV gt 4 infection, and rabbit HEV proved to be transmissible to pigs and cynomolgus macaques[250]. Moreover, pregnant rabbits showed high mortality rates once infected with rabbit HEV, reminiscent of gt 1 infections in humans. Though rabbits may be a good alternative for non-human primates as a model for HEV gt 4, no chronic infection could so far be established. Chronic HEV gt 3 infection was established in the immunodeficient human-liver chimeric mouse model (uPA$^{+/+}$Nod-SCID-IL2Rγ$^{-/-}$) in which livers proved to be infected 2, 6 and 14 weeks after inoculation[246], making this a promising model for future chronic HEV gt 3 studies.

CONCLUSION

In the past 5 years, the knowledge of HEV infection and associated pathogenesis has increased considerably. Only a few years ago, hepatitis E in Europe was described as a travel-related import disease characterized by hepatitis; we now know that HEV is an endemic zoonosis with much broader clinical manifestations. It was classified as a virus inducing acute, transient hepatitis; however, it can also cause chronic infection in the most severely affected patient group – the immune-compromised. Understanding of the hepatitis E virus hide-outs, in the environment, patient populations and within the individual host, is increasing, although additional studies are warranted. Therefore it has become increasingly clear that there is no such thing as *the* hepatitis E phenotype, since the virus comes with a plethora of geographical, epidemiological and clinical 'flavors'.

Chapter 6

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Chapter 7

Nederlandse samenvatting

Hepatitis E virus veroorzaakt per jaar wereldwijd meer dan 3 miljoen acute infecties, met meer dan 70.000 doden. Bij gezonde mensen gaat een infectie van het hepatitis E virus meestal gepaard met milde symptomen, o.a. vermoeidheid. In een sporadisch geval kan de besmetting tot een leverontsteking (hepatitis) leiden. Het virus zorgt voor uitbraken in ontwikkelingslanden, waarbij besmetting optreedt door de feco-orale route, en een hoge sterfte onder zwangere vrouwen veroorzaakt. Enkele decennia geleden, was men van mening dat bovenstaande beschrijving accurate informatie over de epidemiologie van het hepatitis E virus was, en werd het in Nederland vooral als een reizigersziekte beschouwd. Echter sinds een aantal jaren weten we dat een ander genotype (3) van het hepatitis E virus dan hierboven beschreven (genotype 1) vooral endemisch is in Westerse, geïndustrialiseerde landen. De patiënt met een slecht immuunsysteem (immuun- gecompromitteerden), zoals ontvangers van orgaan-transplantaten en patiënten met hematologische maligniteiten, kunnen aan een chronische vorm van hepatitis E lijden. In dit promotieonderzoek is onderzocht welke immuun gecompromitteerde patiënten groepen aan (een chronische vorm van) hepatitis E leden en wat de klinische gevolgen van hepatitis E bij deze groepen patiënten zijn. Daarnaast hebben we op twee manieren (*in vivo* en *in vitro*) de infectiviteit van verschillende lichaamsmaterialen gemeten

HEPATITIS E VIRUS

Hepatitis E virus behoort tot de familie *Hepeviridae*, genus *Orthohepevirus A*. Het heeft geen envelop en is ongeveer 27-34 nanometer groot. Het erfelijk materiaal bestaat uit positief enkelstrengs RNA van ongeveer 7200 baseparen. Het genoom heeft 3 leesramen, ORF1, 2 en 3, waarvan virale eiwitten zoals RNA afhankelijk polymerase en het capside-eiwit worden afgeschreven. Het virus kan op basis van het genoom, epidemiologie en geografische verspreiding worden onderverdeeld in 4 genotypen die mensen kunnen infecteren. Genotype 1 en 2 komen in ontwikkelingslanden voor en veroorzaken daar grote uitbraken, terwijl genotypen 3 en 4 in geïndustrialiseerde landen voorkomen en endemisch zijn. Genotypen 1 en 2 kunnen voor zover we weten alleen mensen infecteren; genotypen 3 en 4 zijn zoönosen, die dus zowel bij mensen als dieren voorkomen. Het grootste reservoir is de varkenspopulatie, maar het virus is ook aangetoond in wilde dieren, zoals herten en wilde zwijnen.

REPLICATIECYCLUS

Er is nog veel onbekend over de replicatie-cyclus van het hepatitis E virus, maar op basis van de karakterisatie van virale eiwitten en bekende replicatie strategieën van positief enkelstrengs RNA virussen, is er toch de volgende hypothese gevormd: het virus hecht aan de buitenkant van de cel met niet-specifieke 'plakkerige' moleculen. Vervolgens zal het virus interactie aangaan met een nog onbekende specifieke receptor, waardoor het virusdeeltje de cel binnen gaat en het erfelijk materiaal wordt uitgepakt. Het positief enkelstrengs RNA kan meteen als sjabloon dienen voor het afschrijven van virale eiwitten, met behulp van de machinerie van de gastheer cel. Het geproduceerde RNA-afhankelijk-RNA-polymerase synthetiseert vervolgens het negatief strengs RNA, dat op zijn beurt weer als sjabloon voor het genomisch viraal RNA kan dienen. De virale eiwitten vormen samen met het virale RNA een nieuw virion, dat de cel verlaat om nieuwe cellen te infecteren.

HEPATITIS E DIAGNOSTIEK

Om de onderzoeksvragen in dit promotie onderzoek goed te kunnen beantwoorden, zijn de juiste diagnostische technieken essentieel. Bij mensen met een goed functionerend immuunsysteem worden direct na een infectie verschillende soorten antistoffen tegen het virus gemaakt. Bij een recente infectie worden IgM antistoffen aangemaakt, terwijl in de herstellende fase juist IgG antistoffen worden gevonden; de techniek waarmee we deze antistoffen kunnen aantonen die tegen het hepatitis E virus gericht zijn, heet ELISA. In hoofdstuk 2 is een aantal commerciële ELISAs, die anti-HEV IgM en IgG , vergeleken. In de literatuur was namelijk bekend dat er grote verschillen waren in gevoeligheid en specificiteit van deze testen. Vervolgens zijn met deze testen ook de verschillen in antistof-reactie in immuun-competente en immuun-gecompromitteerde patiënten gemeten. Uit dit onderzoek bleek, dat de afweerreactie tegen hepatitis E virus van patiënten met een slecht werkend immuunsysteem enorm vertraagd was ten opzichte van patiënten met een normaal immuunsysteem. De conclusie was dat het meten van antistoffen gericht tegen het hepatitis E virus bij immuun-gecompromitteerde patiënten niet aan te bevelen is, maar met moleculaire technieken het virale genoom aangetoond dient te worden.

HEPATITIS E VIRUS IN IMMUUN-GECOMPROMITTEERDE PATIENTEN

Mensen met een verzwakt immuunsysteem, de immuun-gecompromitteerden hebben een verhoogd risico op het krijgen van een chronische hepatitis E. In hoofdstuk 3 is de onderzoeksvraag welke patiënten groepen in Erasmus MC aan hepatitis E leden en wat de klinische gevolgen daarvan waren beantwoord. Hiertoe zijn alle solide orgaan (hart-, long-, nier- en lever-)-transplantatie-ontvangers getest op de aanwezigheid van HEV RNA en antistoffen tegen HEV, waar bij 1% een HEV-infectie werd aangetoond. Dit lijkt een laag percentage, maar de klinische gevolgen van chronische HEV kunnen zeer ernstig zijn (hoofdstuk 3.2). Het blijkt dat een chronische HEV-infectie in een relatief korte periode, vergeleken met andere hepatotrope virussen (HBV/HCV), tot fibrose (littekenvorming) van de lever kan leiden. Fibrose kan leiden tot cirrose en hepatocellulair carcinoma, aandoeningen die levensbedreigend zijn. Van patiënten met hematologische afwijkingen (hoofdstuk 3.3) bleken 2,3% patiënten HEV RNA positief. Alle patiënten waren geïnfecteerd met het genotype 3 en er kon geen gerelateerde (ziekenhuis) besmettingen aangetoond worden. Zoals ook bij andere infectieziekten werd een vertraagde afweerreactie tegen HEV gevonden bij de immuun-gecompromitteerde patiënten, waarbij HEV specifieke IgM antistoffen gemiddeld 4 maanden later meetbaar waren dan HEV RNA. Dit bevestigde onze bevindingen uit hoofdstuk 2. Opmerkelijk is dat HEV-infectie zich vaak niet duidelijk klinisch presenteert. De infectie was ook bij geen van de door ons opgespoorde HEV geïnfecteerde patiënten gediagnosticeerd. Dit is wellicht te wijten aan de tot voor kort bestaande onbekendheid met het feit dat HEV chronische infecties kan veroorzaken en de afwezigheid van diagnostische mogelijkheden om een HEV-infectie aan te tonen. Echter ook met het feit dat patiënten veelal slechts milde leverontstekingen ontwikkelden, met lage transaminase-waarden heeft hier ongetwijfeld toe bijgedragen. Van de hematologische patiënten bleken er twee van de acht een gemiddeld ALT niveau te hebben binnen de normaalwaarden (ULN), bij vier was sprake van een verdubbeld ULN en slechts bij twee patiënten werd een meer dan viervoudig verhoogd ULN gevonden. Aan de andere kant konden de piek-ALT waarden tot meer dan 34 maal het ULN stijgen. Deze factoren leidden tot foutieve diagnoses, zoals toxische lever ziekte (DILI) en afstotingsziekte (GVHD). De juiste diagnose is van groot belang, aangezien de initiële HEV behandeling bestaat uit het verlagen van immuunsuppressie (zodat het immuunsysteem het virus kan klaren), terwijl in andere gevallen bij verhoogde leverenzymwaarden,

zoals bij GVHD, de immuunsuppressie juist verhoogd dient te worden om de afweerreactie tegen te gaan. Het verlagen van deze immuunsuppressie is een delicate balans tussen het voorkomen van afstoting van het transplantaat en het activeren van het immuunsysteem voor virusklaring. Beide (afstoting en klaring) geven een verhoging in ALT waarde, waardoor het bij beperkte diagnostische mogelijkheden voor een behandelend arts erg moeilijk is om in te schatten welk proces gaande is. Deze laatste overweging en omdat niet bij iedere patiënt de immuunsuppressie verlaagd kan worden ivm dreigende afstoting, zijn redenen waarom ook antivirale medicatie, voornamelijk ribavirine, wordt gebruikt voor de behandeling van HEV-infecties in immuun- gecompromitteerde patiënten. Hoofdstuk 3.4 beschrijft de onderliggende ziekten van HEV RNA positieve patiënten in het Erasmus MC, de klinische gevolgen en behandeling van HEV-infecties. Uit deze studie bleek dat de meerderheid van HEV geïnfecteerde patiënten immuun-gecompromitteerd was door solide orgaan transplantatie (SOT). Vooral ontvangers van harttransplantaten hadden een groter risico op een HEV-infectie. Op drie patiënten na, ontwikkelden al deze HEV geïnfecteerde patiënten een hepatitis (verhoogde leverenzym waarden), die bij ongeveer een derde een chronisch beloop had. Zoals ook in de literatuur beschreven, klaarden geen van de SOT ontvangers de HEV-infectie tussen 3-6 maanden spontaan. Met deze informatie, is het wetenschappelijk onderbouwd om niet na 6 maanden (dus alleen chronisch geïnfecteerde patiënten), maar al na 3 maanden te starten met antivirale therapie. Alle 25 patiënten die behandeld werden met ribavirine, hebben de HEV-infectie geklaard. In totaal zijn er vier patiënten overleden met een actieve HEV-infectie en acht patiënten kort nadat ze de HEV-infectie hadden geklaard. Aangezien deze patiënten onderliggende ziekte hadden is het niet mogelijk om de doodsoorzaak aan de HEV-infectie alleen toe te schrijven. Echter, patiënten met bestaande leverziekte hadden significant snellere progressie naar cirrose, wat aan acuut-op-chronische lever falen (ACLF) toegeschreven kan worden.

IN VITRO EN *IN VIVO* MODELLEN VOOR CHRONISCHE HEV-INFECTIES

Om de gevolgen van acute en chronische HEV-infecties te kunnen bestuderen, zijn er geschikte technieken en modellen nodig. Een belangrijke techniek om eigenschappen van HEV te bestuderen is *in vitro* HEV kweek. Hoofdstuk 4 beschrijft de HEV kweek op A549 long adenocarcinoma cellen, waarbij we een verschil van infectiviteit tussen serum en faeces is aan te tonen. Dit wordt bevestigd in het *in vivo* model van immuun deficiënte muizen met een lever die bestaat uit humane en muizen lever cellen. *In vitro* HEV kweek en het chimere muizenmodel zijn een basis om in de toekomst o.a. antivirale therapieën te bestuderen.

Chapter 8

Dankwoord

Tja, en dan ben je aankomen bij het belangrijkste (en meest gelezen) onderdeel van je proefschrift… nu maar hopen dat ik niemand vergeet….mocht dat toch gebeurd zijn, bij deze alvast excuses!
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Allereerst natuurlijk Ab, mijn promotor. De reden waarom ik in de virologie geïnteresseerd raakte tijdens mijn HLO opleiding, was een artikel uit jouw stal. Ik moest een review schijven, o.a. over ISCOMS, waar jij voorvechter van was. Daarom ook maar eens gaan kijken om bij die afdeling stage te gaan lopen, want die virussen waren iets ongrijpbaars wat het enorm interessant maakte. Omdat de uitdagingen bleven komen, heb ik nooit meer een goede reden gehad om weg te gaan. Na een aantal cursussen was jij degene die me aan het denken zetten, "ga nou eens een echte opleiding doen in plaats van al die losse cursusjes". Van het een kwam het ander, je weet mensen te motiveren om door te gaan, de top te bereiken, daar heb ik veel respect voor. Ik ben stiekem toch wel een beetje trots dat het HEV ELISA paper jouw 1000^{e} publicatie is! Dank voor je steun in dit traject.

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En natuurlijk kan ik het beste moleculair diagnostische team van de wereld niet overslaan. Op volgorde van binnenkomst : Jolanda, Judith, Cedric, Chantal, Manon, Mark, Claudia, Sev, Jolanda M, Freek en Anne(ik schoffel je er gewoon even bij)…zonder een fantastisch team is een teamleider nergens, ieder van jullie heeft zijn eigen kwaliteiten, en ik ben erg blij om die te mogen ondersteunen. Ik hoop dat jullie nog lang en gelukkig bij de klinische virologie mogen werken!

Ook de andere mensen van de unit klinische virologie zijn natuurlijk erg belangrijk voor me, Hans, Lilian, Machteld, Monique, Jans, Robin, Eric, Jeroen, Pieter, Corine, Sandra, Janine, Thea, Ada, Georgina, Mikel, Be, Mouring, Cobi, Truus, Marion, Soerani, Helène, Mark P. en Ruud. Speciale dank voor Guido, die de enige echte HEV-kweker is in het land, en natuurlijk Darina, Simone en Loubna

voor jullie eindeloze ondersteuning! Martin, ondanks dat je niet meer bij ons werkt, wil ik je toch graag bedanken voor de jarenlange prettige samenwerking, als ik nog eens wil sparren, dan weet ik je te vinden!

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Chapter 9

About the author

CURRICULUM VITAE

The author of this thesis was born on August 16th, 1976 in Zwijndrecht, the Netherlands. She graduated from high school in 1994 (level "VWO", Gertrudislyceum, Roosendaal) after which she entered medical laboratory school, University West-Brabant, specialization medical microbiology. During this education she did her internship at the department of virology, Erasmus University in Rotterdam on the production of monoclonal antibodies against canine distemper virus and rabies virus specific immunoglobulins of *Phoca vitulina*. After finishing her bachelor in science degree in 1997, she worked as a technician at the department of virology at the Erasmus University, specializing in molecular diagnostic techniques and (antiviral resistance of) hepatitis B virus. She continued to specialize in molecular medicine in 2008 during her Master of science study at the School of Molecular medicine, Erasmus University in Rotterdam. During this master study, she did her research rotation at the department of pathology and finished her master thesis in 2010 at the department of virology entitled "Development of a recombinant virus assay for analysis of phenotypic NRTI resistance in HIV-2". After graduation, she became team leader of molecular diagnostics, unit clinical virology and started her PhD in 2011 under supervision of Prof.dr. A.D.M.E. Osterhaus and Dr. A. van der Eijk. The focus of this project was to study hepatitis E virus in immune-compromised patients of which the results are presented in this thesis.

PHD PORTFOLIO

Name:	Suzan D. Pas
ErasmusMC department:	Department of Viroscience
Research School:	Post-Graduate Molecular Medicine
PhD period:	2011-2015
Promotor:	Prof.dr. A.D.M.E. Osterhaus
Co-promotor	Dr. A.A. van der Eijk

Education

2011 – 2015	PhD program, Erasmus Medical Centre, Rotterdam, The Netherlands. PhD thesis: Hepatitis E virus in immunocompromised patients.
2008 - 2010	Master of Science, Erasmus university, Rotterdam, The Netherlands. Specialization: Molecular Medicine
1994 –1997	Bachelor of Science, Biology and Medical Laboratory Practice, the Avans University in Etten-Leur, the Netherlands. Specialization: medical microbiology.

In depth courses

2012	Management in science, Erasmus MC, Rotterdam, The Netherlands
2012	Teach the teacher III, Erasmus MC, Rotterdam, The Netherlands
2012	Statistics and assay validation, QAeducation, Breda, The Netherlands
2008	Phylogeny, Molecular Medicine School, Erasmus University Rotterdam.
2007	Finding your way in biological information, Molecular Medicine School, Erasmus University, Rotterdam.
2001	SPSS (statistics).

Attended congresses and scientific presentations

2015	Labtechnology, The Netherlands	invited lecture
2015	ESCV, Edinburgh, United Kingdom	oral and poster presentation
2015	LabSafety event, Ede, The Netherlands	invited lecture
2015	NVMM spring meeting, Papendal, The Netherlands,	attended
2015	NVML vakbeurs 2015, Nijkerk, The Netherlands	invited lecture
2014	Lab automation, Nijkerk, The Netherlands	invited lecture
2013	AASLD, Washington, USA	oral presentation
2013	ESCV, Lyon, France	oral presentation
2013	28th Liverday and post-AASLD symposium, Rotterdam, The Netherlands	attended
2013	ECCMID, Hepatitis E virus workshop, Berlin, Germany	invited lecture
2013	EASL, Amsterdam, The Netherlands	poster presentation
2013	NVMM spring meeting, Papendal, The Netherlands	oral presentation
2012	Dutch society hepatology meeting, The Netherlands	oral presentation
2012	27th ErasmusMC Liverday, Rotterdam, The Netherlands	attended
2012	ESAR, Vienna, Austria	invited lecture
2012	EASL, Barcelone, Spain	poster presentation
2012	10th HIV and Hepatitis drug resistance workshop, Barcelone, Spain	invited lecture
2011	7th European Molecular Meeting, Scheveningen, The Netherlands	oral presentation
2011	9th HIV and Hepatitis drug resistance workshop, Cyprus	attended
2009	6th European Molecular Meeting, Scheveningen, The Netherlands	attended
2009	ESCV, Instanbul, Turkey	oral presentation
2007	5th European Molecular Meeting Scheveningen, The Netherlands,	oral presentation
2001	ESCV, Lahti, Finland,	oral presentation
2000	ESCV, Glasgow, Scotland,	poster presentation

TEACHING

2014	WHO Lab-preparedness training
2010 – 2015	Member of the teaching group of clinical microbiologist trainees, supervision of laboratory internships molecular biology.
2010 – 2015	Teacher for Master infection and immunity, Molecular medicine school - Summer course I : "Basic laboratory techniques" - Summer course II : "PCR and real-time PCR" and "Molecular diagnostics in virology" - Biomedical research techniques : "Primers and probes"
1997 – 2015	(Co) Supervision of BSc and MSc students

Specific teaching abroad

2015	Mission leader of the first team in a Dutch Mobile Laboratory for Ebola virus molecular diagnostics, Freetown, Sierra Leone.
2014	Mission leader of a molecular training/lab assessment in Doha, Qatar
2010	Molecular training in Indonesia, Bandung, Hasan Sadikin Hospital.

MISCELLANEOUS

- Scientific expert for QCMD, Glasgow, UK
- Reviewer for several peer-reviewed journals;
  Journal of Clinical Virology, EBiomedicine, Scandinavian Journal of Infectious Diseases, Emerging Infectious Diseases, BMC infectious diseases, European Journal of Clinical Microbiology & Infectious Diseases, Diagnostic Microbiology and Infectious Disease, Journal of Medical Virology, Case reports in Nephrology.

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1. **Pas SD**, Fries E, De Man RA, Osterhaus AD, Niesters HG. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. Journal of clinical microbiology 2000;38:2897-901.

2. Niesters HG, De Man RA, **Pas SD**, Fries E, Osterhaus AD. Identification of a new variant in the YMDD motif of the hepatitis B virus polymerase gene selected during lamivudine therapy. Journal of medical microbiology 2002;51:695-9.

3. **Pas SD**, de Man RA, Fries E, Osterhaus A, Niesters HGM. The dynamics of mutations in the YMDD motif of the hepatitis B virus polymerase gene during and after lamivudine treatment as determined by reverse hybridisation. Journal of Clinical Virology 2002;25:63-71.

4. **Pas SD**, Niesters HGM. Detection of HBV DNA using real time analysis. Journal of Clinical Virology 2002;25:93-4.

5. van der Eijk AA, Niesters HGM, **Pas SD**, de Man R. Persistence of YMDD variants after withdrawal of Lamivudine. Journal of hepatology 2002;36:304-5.

6. Huisman W, Schrauwen EJ, **Pas SD**, Karlas JA, Rimmelzwaan GF, Osterhaus AD. Antibodies specific for hypervariable regions 3 to 5 of the feline immunodeficiency virus envelope glycoprotein are not solely responsible for vaccine-induced acceleration of challenge infection in cats. The Journal of general virology 2004;85:1833-41.

7. van der Eijk AA, Niesters HGM, Hansen BE, **Pas SD**, Janssen HLA, Schalm SW, de Man RA. Paired measurements of quantitative hepatitis B virus DNA in saliva, urine and serum of chronic hepatitis B patients. Journal of hepatology 2004;40:126-.

8. **Pas SD**, Noppornpanth S, van der Eijk AA, de Man RA, Niesters HGM. Quantification of the newly detected lamivudine resistant YSDD variants of Hepatitis B virus using molecular beacons. Journal of Clinical Virology 2005;32:166-72.

9. Sertoz RY, Erensoy S, **Pas S**, Akarca US, Ozgenc F, Yamazhan T, Ozacar T, Niesters HG. Comparison of sequence analysis and INNO-LiPA HBV DR line probe assay in patients with chronic hepatitis B. Journal of chemotherapy 2005;17:514-20.

10. Sertoz RY, Erensoy S, **Pas S**, Niesters HG. [Evaluation of the agreement of results obtained from ABI Prism 7000 and 7700 sequencers in the quantification of hepatitis B virus DNA]. Mikrobiyoloji bulteni 2005;39:175-81.

11. van der Eijk AA, Niesters HGM, Hansen BE, **Pas SD**, Richardus JH, Mostert M, Janssen HLA, Schalm SW, de Man RA. Paired, quantitative measurements of hepatitis B virus DNA in saliva, urine and serum of chronic hepatitis B patients. European journal of gastroenterology & hepatology 2005;17:1173-9.

12. Bosis S, Esposito S, Niesters HG, Tremolati E, **Pas S**, Principi N, Osterhaus AD. Coronavirus HKU1 in an Italian pre-term infant with bronchiolitis. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology 2007;38:251-3.

13. Huisman W, Schrauwen EJA, **Pas SD**, van Amerongen G, Rimmelzwaan GF, Osterhaus A. Evaluation of ISCOM-adjuvanted subunit vaccines containing recombinant feline immunodeficiency virus Rev, OrfA and envelope protein in cats. Vaccine 2008;26:2553-61.

14. Huisman W, Schrauwen EJA, Tijhaar E, Suzer Y, **Pas SD**, van Amerongen G, Sutter G, Rimmelzwaan GF, Osterhaus A. Evaluation of vaccination strategies against infection with feline immunodeficiency virus (FIV) based on recombinant viral vectors expressing FIV Rev and OrfA. Veterinary immunology and immunopathology 2008;126:332-8.

15. **Pas SD**, Tran N, de Man RA, Burghoorn-Maas C, Vernet G, Niesters HG. Comparison of reverse hybridization, microarray, and sequence analysis for genotyping hepatitis B virus. Journal of clinical microbiology 2008; 46:1268-73.

16. Sertoz RY, Erensoy S, **Pas S**, Ozacar T, Niesters H. Restriction fragment length polymorphism analysis and direct sequencing for determination of HBV genotypes in a Turkish population. The new microbiologica 2008;31:189-94.

17. Reijnders JGP, Leemans WF, Hansen BE, **Pas SD**, de Man RA, Schutten M, Janssen HLA. On-treatment monitoring of adefovir therapy in chronic hepatitis B: virologic response can be assessed at 24 weeks. Journal of viral hepatitis 2009;16:113-20.

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19. Veldhuijzen IK, Mes THM, Mostert MC, Niesters HGM, **Pas SD**, Voermans J, de Man RA, Gotz HM, van Doornum GJJ, Richardus JH. An Improved Approach to Identify Epidemiological and Phylogenetic Transmission Pairs of Source and Contact Tracing of Hepatitis B. Journal of medical virology 2009;81:425-34.

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24. Fibriani A, Farah N, Kusumadewi I, **Pas SD**, van Crevel R, van der Ven A, Boucher CA, Schutten M. Low cost HIV-1 quantitative RT-PCR assay in resource-limited settings: improvement and implementation. Journal of virological methods 2012;185:118-23.

25. **Pas SD**, de Man RA, Mulders C, Balk A, van Hal PTW, Weimar W, Koopmans MPG, Osterhaus A, van der Eijk AA. Hepatitis E Virus Infection among Solid Organ Transplant Recipients, the Netherlands. Emerging infectious diseases 2012;18:869-72.

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28. Sonneveld MJ, Rijckborst V, Zeuzem S, Heathcote EJ, Simon K, Senturk H, **Pas SD**, Hansen BE, Janssen HLA. Presence of precore and core promoter mutants limits the probability of response to peginterferon in hepatitis B e antigen-positive chronic hepatitis B. Hepatology 2012;56:67-75.

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39. van Velzen M, Ouwendijk WJD, Selke S, **Pas SD**, van Loenen FB, Osterhaus A, Wald A, Verjans G. Longitudinal study on oral shedding of herpes simplex virus 1 and varicella-zoster virus in individuals infected with HIV. Journal of medical virology 2013;85:1669-77.

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45. Hassing RJ, van der Eijk AA, Lopes VB, Snijdewind IJ, de Man RA, **Pas SD**, van der Ende ME. Hepatitis E prevalence among HIV infected patients with elevated liver enzymes in the Netherlands. Journal of Clinical Virology 2014;60:408-10.

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49. Prachayangprecha S, Schapendonk CM, Koopmans MP, Osterhaus AD, Schurch AC, **Pas SD**, van der Eijk AA, Povoorawan Y, Haagmans BL, Smits SL. Exploring the potential of next-generation sequencing in diagnosis of respiratory viruses. Journal of Clinical Microbiology 2014.

50. Erdin BN, **Pas SD**, Durak I, Schutten M, Sayimer AA. A 5-year study of adenoviruses causing conjunctivitis in Izmir, Turkey. Journal of Medical Virology 2015;87:472-7.

51. Brouwer WP, van der Meer AJ, Boonstra A, **Pas SD**, de Knegt RJ, de Man RA, Hansen BE, ten Kate FJ, Janssen HL. The impact of PNPLA3 (rs738409 C>G) polymorphisms on liver histology and long-term clinical outcome in chronic hepatitis B patients. Liver international : official journal of the International Association for the Study of the Liver 2015;35:438-47.

52. Smits SL, Raj VS, **Pas SD**, Reusken CB, Mohran K, Farag EA, Al-Romaihi HE, AlHajri MM, Haagmans BL, Koopmans MP. Reliable typing of MERS-CoV variants with a small genome fragment. Journal of Clinical Virology 2015;64:83-7.

53. Vanwolleghem T, Hou J, van Oord G, Andeweg AC, Osterhaus AD, **Pas SD**, Janssen HL, Boonstra A. Re-evaluation of HBV clinical phases by system biology identifies unappreciated roles for the innate immune response and B cells. Hepatology 2015 Mar 22. doi: 10.1002/hep.27805.

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63. Hundie GB, Raj VS, Michael DG , MD, **Pas SD**, Osterhaus AO, Koopmans MP, Smits SL, Haagmans, BL. Molecular Epidemiology and Genetic Diversity of Hepatitis B Virus in Ethiopia, submitted.

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65. **Pas SD**, Arends P, Voermans J, Schürch AC, Zoutendijk R, Jackson K, Haagmans BL, Smits SL, Boucher CAB, van der Eijk AA, Locarnini S, Janssen HLA. Ultra-deep pyrosequencing in Lamivudine naïve chronic hepatitis B patients developing Entecavir resistance and virologic breakthrough, submitted.

66. van de Garde MDB, **Pas SD**, van der Net G, Osterhaus ADME, Haagmans BL, Boonstra A, Vanwolleghem T. Hepatitis E virus genotype 3 infection of human liver chimeric mice and A549 cells as models for chronic HEV infection, submitted.

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