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Swine-like hepatitis E viruses are a cause of unexplained hepatitis in the Netherlands

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SUMMARY. Hepatitis E virus (HEV) infections in developed countries are recognized as an imported disease related to travel to endemic regions. However, increasing evidence suggests that HEV infection may also occur in the developed countries and that swine may act as a possible reservoir. To investigate the indigenous transmission of HEV in the Netherlands, sera from 50 blood donors and 1027 sera from patients with acute hepatitis were screened with an ELISA for HEV-specific IgG and IgM. Because the Netherlands is considered a nonendemic region, all positive ELISA results were confirmed by immunoblot to exclude false-positive results. Evidence of recent HEV infection was detected in 0% of the blood donors and 4.4% of the cases, based on combined positive IgM and IgG responses. The serodiagnosis was confirmed by a positive polymerase chain reaction (PCR) in 24 patients with hepatitis (2.3% overall, 51% of confirmed IgM+/IgG+ cases). IgG antibodies alone were detected in 4.2% of patients. We found related sequences to virus strains detected in Dutch pigs (genotype 3, 91–97% homology) in 89% of PCR-confirmed HEV patients. The detection of unique swine-like HEV sequences in 16 indigenous hepatitis patients without a recent travel history suggests that HEV is endemic in the Netherlands. We recommend including HEV tests in unexplained acute hepatitis patients, despite their travel history.

Keywords: anti-hepatitis E, hepatitis E virus, reverse transcriptase-polymerase chain reaction, seroprevalence, the Netherlands.

INTRODUCTION

In the Netherlands, the most commonly diagnosed causes of acute viral hepatitis infections are the hepatitis A (HAV), B (HBV) and C (HCV) viruses, cytomegalovirus (CMV) and Epstein–Barr virus (EBV) infections. Hepatitis E virus (HEV), another recognized pathogen that causes acute viral hepatitis, is mainly considered an imported disease [1,2]. However, increasing evidence suggests that indigenous HEV infection may also occur in developed countries with a high standard of living [3–7]. We recently identified a cluster of two confirmed cases and one presumed case of hepatitis associated with HEV reported in persons without a travel history in the north of the Netherlands [6]. The investigation of a larger group of hepatitis patients from the same region, who tested negative for HAV, HBV and HCV, and with no known history of foreign travel, also provided evidence for locally acquired hepatitis E in the Netherlands [8].

Hepatitis E is typically a self-limiting disease of varying severity, presenting as acute icteric hepatitis with clinical symptoms similar to those of hepatitis A. HEV infections have been reported over a wide geographical area in Asia, Africa, the Middle East and central America [9]. The overall case/fatality rates are 0.5–3%, but may be much higher in pregnant women (15–25%) [10,11]. HEV is transmitted primarily by the faecal–oral route [12]. While the route of transmission in the developed countries remains unclear, the transmission of HEV was recently correlated with eating raw or undercooked wild boar and deer meat in Japan [13–15]. Molecular evidence for natural HEV infection in swine has been reported in both HEV-endemic and -nonendemic countries worldwide, including the Netherlands [16–20], and the prevalence of antibodies against HEV was found to be higher among persons working with swine than among the control groups [18,21–23]. There is also evidence for

Abbreviations: HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; CMV, cytomegalovirus; EBV, Epstein–Barr virus; RT-PCR, reverse transcriptase-polymerase chain reaction.

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transmission by blood transfusions [24–27]. Asymptomatic cases have also been described [10].

Hepatitis E virus is a nonenveloped, single-stranded RNA virus of positive sense (7.2 kb) and is classified into its own group of Hepeviridae [28]. The HEV strains known to infect humans fall into at least four groups, namely genotype 1 (Asia and Africa), genotype 2 (Mexico), genotype 3 (the United States and Europe) and genotype 4 (China, Taiwan and Japan). Genotype 3 and 4 strains have been detected in pigs, with genotype 3 distributed worldwide [15,29,30].

Because viraemia is thought to be present in the serum only during the acute phase of illness, the diagnosis of an HEV infection is mainly dependent on serology [31]. Both HEV-specific IgM and IgG are generally detectable at the onset of disease. The titres of IgM decline rapidly during early convalescence but can be detected in some patients for 5–6 months [32]. IgG can be detected in most patients for at least 1 year after acute infection. The seroprevalence in blood donors in the nonendemic countries is low (1–3%) and has been controversially assumed to be false positive [2,33–35].

To investigate whether locally acquired HEV infections might be overlooked in the Netherlands, we investigated groups of patients with acute non-A, -B or -C hepatitis and without recent travel history from different regions in the Netherlands and sera submitted for HEV and HAV diagnostics by serology and molecular methods.

MATERIALS AND METHODS

Study populations

The specificity of the HEV serology was calculated using serum samples from 50 randomly collected healthy blood donors from the Netherlands. Requests were sent to three regional laboratories that provide routine diagnostic services for common infectious diseases. The laboratories were located in different regions of the country. Samples had been collected between 2002 and 2004. Laboratories were asked to collect sera from patients with locally acquired acute hepatitis of possible viral aetiology that tested negative for known causes of viral hepatitis (HAV, HBV, HCV, CMV and EBV with no known travel history). For logistical reasons, one of the laboratories (Terneuzen) included all specimens that were submitted for HAV IgM serology. Samples from a total of 504 patients were included in the study (Groningen, $n = 209$; 's-Hertogenbosch, $n = 100$ and Terneuzen, $n = 195$). An additional 523 hepatitis cases that had been submitted for HEV diagnosis to our laboratory between 2002 and 2005 were examined for HEV-specific IgM and IgG.

IgG/IgM hepatitis E virus ELISA

The HEV-specific IgG and IgM ELISAs were performed by commercially available assays according to the manufacturer's instructions (Genelabs Diagnostics Inc., Redwood City, CA, USA). The antigens used in the assays were recombinant proteins from the ORF2 and ORF3 regions of HEV serotypes 1 and 2 (Burmese and Mexican strain variants).

Positive and negative control samples provided with the kit were included in each run. In addition, an internal low-positive IgG or IgM sample was tested to control for intra-assay variation. Cut-off values were calculated as 0.500 (for IgG) or 0.400 (for IgM) plus the mean absorbance of the nonreactive controls. Ratios of ≥1 (OD value of the test sample divided by the cut-off) were considered positive and were further analysed by immunoblot and PCR. The specificities of the HEV-specific ELISAs indicated by the manufacturer were 97% and 98% for IgM and IgG, respectively, in nonendemic and 96% and 85% in endemic regions. The sensitivity of the IgM ELISA was reported to be 93% if the patient was tested within 14 days after the onset of disease.

IgG/IgM hepatitis E virus recomBlot

Because the Netherlands is considered a nonendemic region, positive ELISA results might have reflected false-positive results. Therefore, all positive samples were further analysed by a commercially available immunoblot assay to confirm reactivity. The recomBlot uses recombinant antigens that are separated by gel electrophoresis and transferred to a nitrocellulose membrane (Mikrogen; Martinsried, Germany). Antigens on the immunoblot are the N-terminal part of the capsid antigen (GST fusion protein O2-N, 50 kDa), the C-terminal part of the capsid antigen (triple band) (O2-C, 38–41 kDa), the middle part of the capsid antigen (O2-M, 28 kDa) and the ORF3 protein (O3, 15 kDa) of genotypes 1 and 2. The HEV immunoblot was performed according to the manufacturer's instructions. Prior to testing, sera were depleted of IgG with Gullsorb (Meridian Bioscience; Boxtel, The Netherlands) to prevent possible inter-isotype competition and false-positive results caused by rheumatoid factors. An internal low-positive IgM control sample was also included during each run. Sensitivity of the HEV recombinant blot was 85.7% and 97.5% for IgM and IgG, respectively, as determined in acute HEV patients from Madras and stated by the manufacturer. Specificity was reported by the manufacturer as being 100% and 94.9% in the nonendemic region for IgM and IgG, respectively, as reported. In endemic regions, specificity calculated in healthy controls was 98.5% for IgM and only 56% for IgG detection as a diagnostic for recent infection, indicating high exposure to HEV in the general population in these regions reflecting past (asymptomatic) HEV infection. To reduce the likelihood of false-positive results, serum samples were considered serologically positive only when the ELISA result was confirmed by the western blot assay. For this, samples with immunoblot scores >3 for IgG or >5 for IgM were considered positive. Samples with a score labelled as 'borderline' according to the manufacturer (IgM score of 5 and IgG score of 3) were considered to be negative in this study.

Detection of hepatitis E virus RNA in serum samples

We determined the presence of HEV RNA in serum samples as described previously [3,6]. In brief, RNA was extracted from 140 μL of a 10% stool suspension or from serum using commercial columns (QIAamp; Qiagen, Venlo, The Netherlands). Extracted RNA was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with primer pairs corresponding to the ORF2 region followed by Southern hybridization. Used primers were ORF2-s1: 5′-GAC AGA ATT RAT TTC GTC GGC TGG-3′ (sense) and ORF2-a1: 5′-CTT GTT CRT GYT GGT TRT CAT AAT C-3′ (antisense). In total, 40 amplification cycles (1 min at 94 °C, 1 min 30 sec at 55 °C and 1 min at 74 °C each) were performed. Specimens showing a band of expected size (197 bp) on hybridization were considered to be positive. All positive samples were further analysed by sequencing and phylogenetic analyses. The sequences determined in the present study have been deposited in the GenBank nucleotide database (accession numbers: DQ200273–DQ200296). Individual sequences of a 148-bp long fragment were compared with those in the GenBank nucleotide database.

RESULTS

Seroprevalence of hepatitis-E-virus-specific IgG and IgM in blood donors

To estimate the specificity of the combined testing regime of ELISA and immunoblot, a total of 50 serum samples from blood donors were examined for HEV-specific IgM and IgG. Nine samples tested positive in the IgM ELISA (18%) and three samples were positive for IgG (6%). None of the blood donors tested positive for both IgG and IgM HEV-specific antibodies in the ELISA. All reactive samples were tested by IgG and IgM immunoblot irrespective of previous IgG or IgM responses, including samples with a ratio just below 1. None of the samples tested positive for IgM by the immunoblot (Table 1). Of the three IgG positive ELISA results, two were confirmed by immunoblot and one blood donor had a positive immunoblot result for IgG (score 4) after being previously selected for a positive IgM result in the ELISA (Table 1). Three blood donors had low IgG immunoblot scores (4, 4 and 6). Thus, the combination of the ELISA and the immunoblot led to a specificity of 100% and 94% for IgM and IgG serology, respectively.

Seroprevalence of hepatitis-E-virus-specific IgG and IgM in non-A, -B, -C patients with acute (viral) hepatitis with no other known cause of infection and travel history

A total of 309 patients selected for negative serology for other known viral causes of acute hepatitis and no known travel history to HEV endemic regions were tested.

A total of 25 patients (8%) tested positive for IgM by ELISA, but only eight of these (3% of the total, 34% of all ELISA positives) could be confirmed by immunoblot. Twenty patients were positive for IgG only, of which 18 (6% of the total) could be confirmed (Table 1). In this group, six (2%) patients had low IgM levels under the cut-off value by immunoblot (score 3–5). Ten patients were reactive in both the IgM and IgG ELISA, nine of which were confirmed by immunoblot (3% of the total). PCR testing of the seropositive samples yielded six positives. In all of these cases, both IgM and IgG antibodies were detected by immunoblot (Table 2).

Seroprevalence of hepatitis E virus IgG and IgM in patients sent in for hepatitis A virus serology

A total of 195 samples from patients submitted for HAV serology were screened. In this group, two cases of acute HAV infection were diagnosed by a positive HAV-specific IgM response. Nine patients (5%) were positive for HEV-specific IgG alone after confirmation by immunoblot (Table 1). Five of these had high IgG responses (immunoblot score ≥7) but all had low IgM levels (≤3). After immunoblot confirmation, two patients tested positive for both IgM and IgG antibodies. The serodiagnosis of acute HEV infection was confirmed by a positive PCR in one of these patients.

Table 1 Anti-HEV IgM and IgG and PCR results in blood donors, non-A, -B, -C, HAV- and HEV-suspected patients

	Number positive*/total number (%)			
	Only IgM+	Only IgG+	Both IgM+ and IgG+	PCR
Blood donors	0/50 (0)	3/50 (6)	0/50 (0)	0/50 (0)
Acute hepatitis patients				
Non-A, -B, -C	8/309 (3)	18/309 (6)	9/309 (3)	6/309 (1.9)
HAV	2/195 (2)	9/195 (5)	2/195 (1)	1/195 (0.5)
HEV	2/523 (0.4)	33/523 (6)	34/523 (7)	17/523 (3.3)
Total	12/1027 (1.2)	43/1027 (4.2)	45/1027 (4.4)	24/1027 (2.3)

*Serum samples were only considered serologically positive after confirmation of a positive ELISA result with the immunoblot assay.

Table 2 Serological and molecular laboratory results of PCR-positive HEV patients from the non-A, -B, -C, HAV- and HEV-suspected patient groups

	ELISA (ratio)		Immunoblot (total score)		
Patient number	IgM	IgG	IgM	IgG	Sequence data
Non-A, -B, -C patients					
DB26	**6.68**	0.99	**11**	**5**	Swine HepE NLSW 85 97/99 (97%)
DB31*	**2.12**	**6.43**	**9**	**12**	HepE virus ORF1, 166/173 (95%) X99441 (Madras, India 1993)
DB45	**1.08**	**4.17**	**10**	**12**	Swine HepE NLSW 85 97/99 (97%)
GR150	**2.51**	**3.11**	**8**	**10**	Swine HepE NLSW 105 82/89 (92%)
GR174	**2.39**	**3.60**	**7**	**11**	Swine HepE NLSW 36 86/91 (94%)
GR217	**1.02**	**1.91**	**11**	**12**	Swine HepE NLSW 82 167/172 (97%)
HAV patients					
1572	**7.24**	**1.44**	4	**6**	Swine HepE NLSW 105 63/68 (92%)
HEV patients					
02-1650*	**5.81**	**7.00**	**11**	**11**	Nepal NCBI 188/198 (94%)
02-2520	**6.14**	0.77	4	**7**	Swine HepE NLSW 105 138/147 (93%)
03-366	**7.56**	**6.70**	**11**	**12**	Swine HepE NLSW 82 165/172 (95%)
03-986	**7.18**	**6.62**	**11**	**11**	Swine HepE NLSW 105 74/79 (93%)
3796	**4.08**	**1.04**	**8**	**9**	Nepal HepE TK15/92 141/148 (95%)
04-684	**8.53**	**7.44**	**12**	**12**	Swine Hep E NLSW 105 (91%)
04-906	0.47	**1.96**	**8**	**12**	Nepal TK15/92 (96%)
04-1232	**6.45**	**6.25**	**11**	**12**	Swine HEV 123/132 (93%)
05-182	**9.28**	**3.73**	**8**	**8**	Swine NLSW 105 146/157 (92%)
05-397	0.57	0.49	**5**	**7**	Swine HepE NLSW
05-440	0.42	**7.10**	**9**	**12**	Nepal HEV 170/177 (96%)
05-551	0.23	**7.05**	**9**	**12**	Nepal HEV 172/177 (97%)
05-756	**9.35**	**6.56**	**8**	**12**	Swine HEV 152/163 (93%) NLSW 105
05-825	**2.22**	0.12	**7**	**8**	Swine HEV NLSW 82 134/140 (95%)
05-914	0.29	**5.63**	**10**	**12**	HEV TK15/92 150/156 (96%)
05-915	0.06	**7.10**	**7**	**12**	HEV TK15/92 170/178 (95%)
05-1169	**2.23**	**5.09**	**6**	**10**	Swine HEV 157/163 (95%)

Bold indicates positive results.
*No travel reported.

Seroprevalence of hepatitis E virus IgG and IgM in patients sent in for hepatitis E virus serology

A total of 523 patients submitted to our laboratory for HEV serology between 2002 and 2005 were tested. IgM-and IgG-positive results were obtained after confirmation by immunoblot in 7% of the samples ($n = 34$). Only IgM or IgG responses were detected in 0.4% ($n = 2$) and 6% ($n = 33$) of the cases, respectively. The serodiagnosis could be confirmed by a positive PCR in 3.3% of the patients ($n = 17$). All except one of these were dually positive for IgM and IgG (Table 2).

Polymerase chain reaction sequence data

A total of 24 patients tested positive by PCR. Partial capsid sequencing showed that 16 of these patients were infected with swine-related strains (genotype 3) with homology between 91% and 97% to virus strains detected in pigs from the Netherlands [16]. Strains belonging to genotype 1 were detected in the other eight patients. In six of the eight geno type 1 patients, a travel history to the endemic regions was reported, while no travel was reported by the remaining two. The percentage of swine-related HEV strains detected in patients sent into the laboratory with no reported travel history was 89% (16/18) (Table 2).

Figure 1 shows a dendrogram of the HEV sequences detected in the patients and pigs from the Netherlands with sequences of representative strains for genotypes 1, 2, 3 and 4 from the GenBank nucleotide database.

DISCUSSION

Although Zaaijer *et al.* reported two patients with positive HEV serology and without travel history in a group of 21

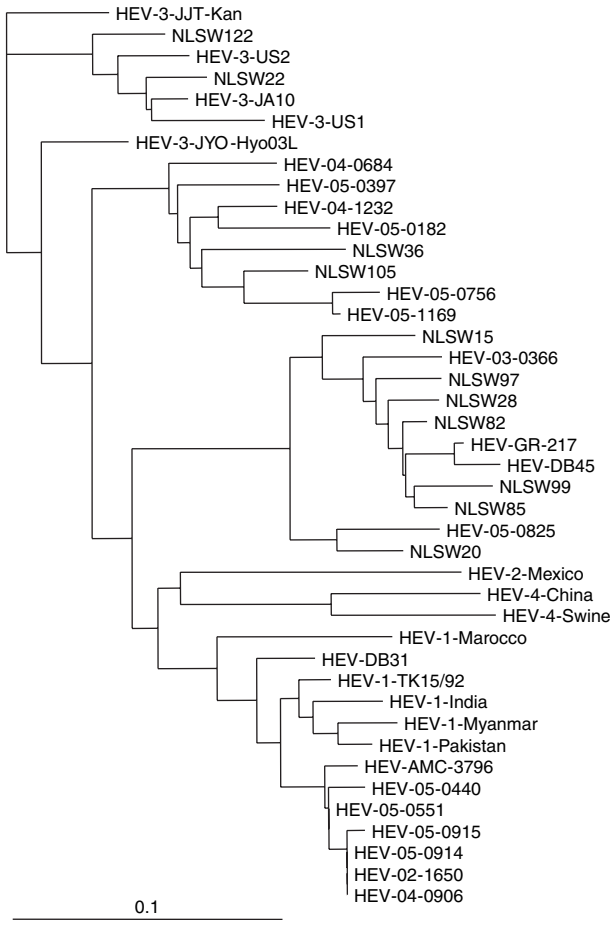

Fig. 1 Phylogenetic comparison of the HEV strains detected in acute hepatitis patients from the Netherlands. Sequences derived from patient samples were compared with the closest matching sequences from GenBank and selected reference strains. A tree was produced using the neighbour-joining method after multiple sequence alignment of a 148-nt long fragment from the capsid gene. The sequences we used were HEV-3-JJT-Kan (AB091394), HEV-3-JA10 (AB089824), HEV-3-US1 (AF060668), HEV-3-US2 (AF060669), HEV-3-JYO-Hyo03L (AB189075), HEV-4-China (AJ272108), HEV-1-India (AF076239), HEV-1-Marocco (AY230202), HEV-2-Mexico (M74506), HEV-1-Myanmar (D10330), HEV-1-TK15/92 (AF051830), HEV-1-Pakistan (M80581), HEV-4-Swine (AY594199), NLSW122 (AF336299), NLSW105 (AF336298), NLSW99 (AF336297), NLSW97 (AF336296), NLSW85 (AF336295), NLSW82 (AF336294), NLSW36 (AF336293), NLSW28 (AF336292), NLSW22 (AF336291), NLSW20 (AF336290), NLSW15 (AF332620), all from GenBank. Sequences from this study are labelled: HEV-GR-217, HEV-02-1650, HEV-03-0366, HEV-04-0684, HEV-04-0906, HEV-04-1232, HEV-05-0182, HEV-05-0397, HEV-05-0440, HEV-05-0551, HEV-05-0756, HEV-05-0825, HEV-05-0914, HEV-05-0915, HEV-05-1169, HEV-DB31, HEV-DB45, HEV-AMC-3796.

Dutch patients [1], HEV is mainly considered an imported disease in the Netherlands from travellers and emigrants returning from known HEV endemic countries. We found clear evidence of HEV infection in acute hepatitis patients with no travel history both by serology and molecular methods.

A positive RT-PCR is the ultimate proof of an acute HEV infection. However, the virus can only be detected in the early phases of the disease and in many cases detection depends on serodiagnosis. Our data show that having a combined IgG and IgM response is a strong indication of a recent HEV infection, as 51% of these patients ($n = 45$) were viraemic.

We detected HEV-specific IgM in combination with IgG in 4.4% of the examined acute hepatitis patients. A similar seroprevalence (5%) was reported among non-A, -B, -C hepatitis patients in the United States [36]. None of the patients with a positive IgM response alone were confirmed by PCR. In these patients, a second follow-up serum sample should be analysed to exclude false positivity.

An additional 4.2% of cases were only IgG positive. This could represent past or recent infection. We detected high IgG responses (immunoblot score ≥7) in combination with low-level IgM (score 3–5) in 1.5% of the patients. As one of these was PCR positive, high-level IgG may indicate recent contact with HEV (especially when samples were taken late after the onset of disease). This also shows that early sample taking may be crucial for the proper diagnosis of an acute HEV infection. Low-level IgG responses (2% of patients) may reflect past exposure or even false positivity.

Remarkably, 6% of a small group of blood donors tested positive for IgG. This is much higher than the previously reported 0.4% positivity detected by ELISA in a group of 1275 blood donors from the Netherlands, but is comparable to those reported from blood donors in the USA, Japan and several other countries [2,33,36,37]. Two of the IgG-positive blood donors had a score just above the cut-off level in the immunoblot (score 4). These low responses could reflect antibody decay over time or false positivity rather than a past HEV infection. In general, low IgG responses of 4 are not detected in acute HEV patients (Table 2). If these two low IgG results are excluded, there is no significant difference between the previously reported 0.4% [2].

In 16 patients, we detected virus strains belonging to the genotype 3 group with homology of 91–97% to virus strains detected in pigs from the Netherlands [16]. Direct transmission of HEV from pigs to humans has been described, but in our data set all patients have unique sequences distinct from porcine HEV strains. The high similarities between human and porcine HEV strains do suggest that cross-species transmissions occur, but we did not find formal proof of such a link. Further research focused on a detailed case follow-up, aimed at pinpointing what the sources of HEV infection are in our country, are presently underway.

Our study also shows that HEV cases could be underreported if physicians consider HEV infection to be a travel-related disease only. Requests for HEV tests are not always part of the routine evaluation of patients who acquired acute HEV without having a history of recent travel. In order to get a first indication of the possible level of underreporting, we examined a group of patients originally suspected of having HAV infection. In this group, an equal number of HAV and HEV cases were diagnosed. While this was only a pilot study, these data indicate that the incidence of (unrecognized) HEV infections may be in the same range as that of HAV. Clearly, more work is needed to address this issue. Approximately, 400 cases of acute HAV infection were reported in 2002 in the Netherlands [38].

Combined with the results of the molecular strain characterization, the data suggest that HEV is endemic in the Netherlands. We state that HEV is a cause of unexplained hepatitis in the Netherlands and therefore recommend that HEV infection should be investigated in all patients with unexplained acute hepatitis, despite their travel history.

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