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Hepatitis E virus risk profile

Identifying potential animal, food and water sources for human infections

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Abstract

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Hepatitis E virus (HEV) was shown to be present in the Netherlands in animals (pigs, wild boar and red deer), food (pig liver, oysters and mussels) and surface water. Locally acquired hepatitis E may therefore be contact-, food- or water-related.

Hepatitis E virus infections are acquired in the Netherlands, but epidemiological studies have failed to identify the sources of those infections thus far. Amongst others because of the incubation period that is too long for tracing studies.

In the Netherlands, most HEV infections remain unnoticed, because of the general mild symptoms of infected individuals. However, in the vulnerable population like the immunocompromised and people suffering from pre-existing diseases, severe hepatitis due to HEV is more common. Individuals in these risk groups may become chronically infected by the virus or may die because of hepatitis. By assessing the contribution of the different potential sources and transmission routes to the exposure of humans to HEV, the more and less important ones may be identified. Intervention measures may be formulated to contain the spread of HEV to humans by restraining the most important transmission routes.

This report describes the sources of HEV that have been detected worldwide. This demonstrated that the HEV-variant that causes hepatitis E in humans is commonly detected in pigs and wild boar. The RIVM studied potential sources of HEV in the Netherlands. Identified sources of HEV were, besides domestic pig and wild boar, also red deer, oysters, mussels, surface water and source water used for drinking water production. Therefore, transmission of HEV may not only be contact- or food related, but may also be water related. These data will be used in a risk assessment model to estimate the exposure of humans to HEV.

Key words:
hepatitis E virus, transmission, zoonosis, human, animal, food, water, the Netherlands

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Rapport in het kort

Hepatitis E virus risicoprofiel
Identificatie van mogelijke dier-, voedsel- en waterbronnen voor humane infecties

In Nederland is het hepatitis E virus (HEV) aangetoond in dieren (varkens, wilde zwijnen, herten), voedsel (varkenslever, oesters, mosselen) en oppervlaktewater. Het is mogelijk dat de verspreiding van HEV uit deze bronnen naar mensen gerelateerd is aan contacten tussen mensen en dieren, aan de consumptie van voedsel of drinkwater, of door in oppervlaktewater te recreëren. Dit blijkt uit onderzoek van het RIVM naar de bronnen en verspreidingsroutes van het HEV-type dat in Nederland voorkomt. Deze gegevens zullen worden gebruikt voor blootstellingsschattingen, die kunnen helpen om gericht interventiemaatregelen te nemen om eventuele risico's voor de volksgezondheid te verlagen.

HEV-infecties kunnen ontstekingen aan de lever veroorzaken, vooral bij mensen met een verminderd afweersysteem. Bij deze risicogroepen kan dit tot chronische leverinfecties leiden of zelfs tot de dood. Bij gezonde mensen lijken HEV-infecties evenwel onopgemerkt te blijven vanwege het milde verloop.

In Nederland worden HEV-infecties doorgaans opgelopen zonder dat de precieze bron kan worden vastgesteld, onder andere vanwege de lange incubatieperiode. Door de bijdrage van de mogelijke bronnen en bijbehorende verspreidingsroutes aan HEV-infecties bij mensen te schatten, kunnen maatregelen worden opgesteld die verspreiding van het virus naar mensen tegengaan.

Uit het onderzoek blijkt dat de HEV-variant die bij de mens hepatitis E veroorzaakt wereldwijd vaak bij varkens en wilde zwijnen voorkomt. Het RIVM heeft vervolgens eventuele bronnen van HEV in Nederland onderzocht. Het blijkt dat niet alleen wilde zwijnen en varkens, maar ook herten, oesters, mosselen en oppervlaktewater dat wordt gebruikt voor recreatie en drinkwaterproductie HEV kunnen bevatten.

Trefwoorden:
hepatitis E virus, transmissie, zoönose, mens, dier, voedsel, water, Nederland

Contents

Summary

Hepatitis E virus infections are acquired in the Netherlands, but epidemiological studies have failed to identify sources leading to those infections. The incidence (*i.e.*, hospitalized cases) of ~10 cases of hepatitis E per year appears to be relatively low, but it may be more commonly present than is currently acknowledged. Specific risk groups seem to be predisposed for a severe form of HEV infection that may result in death. To protect public health, it is therefore important to identify the HEV sources, the associated transmission routes to humans and their contribution to HEV infection.

Epidemiological studies lack the discriminatory power to identify potential risk factors, because of the low number of hepatitis E cases. By quantitative microbiological risk assessment (QMRA), exposure through different transmission routes may be estimated without observed human infections. Risk assessment consists of four components: 1) a risk profile, 2) an exposure assessment, 3) a dose-response model, and 4) the risk characterization. The current report describes the risk profile for HEV. This profile includes a broad overview of existing literature as well as an environmental survey of potential sources of HEV in the Netherlands to gain insight into the Dutch situation.

The literature shows that domestic pigs, wild boar and wild deer are potential animal HEV sources for human infection. Direct contact with pigs and consumption of un(der)cooked meat or organs from these animals have been postulated as potential routes of transmission. In other animals, including cattle, horses, sheep, goats, dogs, cats and rodents the presence of HEV-specific antibodies was described, but further studies are required to determine whether these animals are indeed able to replicate the virus and transmit it to subsequent hosts.

The risk profile identified several data gaps for a full quantitative risk assessment for HEV. The most important identified gap was a lack of knowledge of potential animal HEV sources in the Netherlands other than domestic pigs, the potential environmental HEV sources other than animals, and the potential food- and waterborne transmission routes in the Netherlands. To be able to accurately assess the presence of HEV in the Netherlands, further improvements of the HEV detection assays were established, which are described in this report. Besides domestic pigs and wild boar, the following potential HEV sources have been identified: red deer, oysters, mussels, surface water and source water for drinking water production. Transmission to humans could in theory follow direct-contact from handling animals or animal products, consumption of meat or organs from infected animals, water recreation and the consumption of unboiled tap water. As a next step in the risk assessment, the exposure of humans to HEV will be assessed for the consumption of wild boar, deer meat and unboiled drinking water that is produced from surface water and for direct contact with domestic pigs. The results may subsequently direct the development of intervention measures that effectively reduce the exposure of humans to HEV.

1 Introduction

In 1992, HEV infection was reported for the first time in the Netherlands in patients who contracted the infection presumably in Bangladesh and Pakistan (Van der Pal and Jansen, 1992; Van Zeijl et al., 1992). In these days, HEV was considered to be an imported disease in the Netherlands. A serosurvey among blood donors in the Netherlands, however, yielded an estimated seroprevalence of about 1% (Zaaijer et al., 1993), with one of the seropositive individuals not having traveled internationally in the months preceding blood donation. This finding suggested (a) local HEV source(s) in the Netherlands. This hypothesis was confirmed in 2003, when a cluster was observed of three hepatitis E cases without international travel during three months before onset of symptoms (Widdowson et al., 2003). All patients lived within 10 km from each other, which suggests a common source for infection. Despite retrospective interviews of the patients and investigation into the drinking water quality no HEV source was identified. A subsequent study with 209 acute non-ABC hepatitis cases from the same geographical area suggested that locally-acquired hepatitis E cases occurred more often in that region (Waar et al., 2005). Hence, HEV infections contracted in the Netherlands have been reported occasionally, but may be more commonly present than is currently acknowledged.

About 2% of the Dutch population in the age of 20 up to 65 is estimated to be seropositive for anti-HEV antibodies (Bouwknegt et al., 2008a), and about two third of the observed acute hepatitis E cases in the Netherlands is suggested to be unrelated to travel to endemic countries (Herremans et al., 2007). In the period 1998-2001, about 10 million people in the Netherlands fell within the age-class of 20–65. These data suggest that roughly 130,000 HEV infections were acquired in the Netherlands until 1998-2001 (the period in which the samples from the general population were collected). In case of life-lasting immunity against HEV and no age-effect in the probability of acquiring HEV infection, then about 2,000 HEV infections had been acquired annually by individuals that fall within the age category of 20–65, or 2 per 10,000 persons. Given this estimated HEV incidence, and the finding by Borgen et al. (2008) of on average 7 cases per year that seek medical attention (incidence of ~0.007 per 10000 persons), the majority of infections likely remain unnoticed or run a mild course. However, especially for the risk groups for HEV, possibly men >50 years of age and immunocompromised individuals, infection can be severe, leading to hospitalization and possibly death (Kraan et al., 2004). Therefore, it is important to assess the contribution of the different potential transmission routes to the exposure of humans to HEV.

Epidemiological studies lack the discriminative power to identify potential risk factors in case of a rare disease or diseases with long incubation periods. Because the low number of hepatitis E patients a limited number of cases is available for attribution studies. An approach to study potential exposure routes that are independent of clinical human infections is by quantitative microbiological risk assessment (ILSI Risk Science Institute Pathogen Risk Assessment Working Group, 1996). Risk assessment involves structured modeling of (a) possible transmission route(s) for a pathogen from source up to exposure to humans. Firstly, a risk profile is to be compiled that summarizes all available (and appropriate for the risk assessment) information regarding the pathogen of concern. Secondly, factors that influence pathogen concentration and pathogen ingestion are quantified from data and joined in a mathematical model to estimate the dose being exposed to. Thirdly, the hazard which a pathogen poses is quantified, usually by constructing a dose-response model. And fourthly, the estimated dose from the second step can be related to the dose-response model from the third step, yielding an estimate of the infection risk. The estimated infection risks per route can subsequently be

compared, identifying those route(s) leading to the highest HEV infection risk. These findings may subsequently be used to develop intervention measures that effectively reduce the HEV incidence among humans.

The current report presents the risk profile for HEV in the Netherlands, identifying potential food, water and animal sources (chapter 2). Issues regarding the detection of HEV are discussed in chapter 3. The data gaps identified during compilation of the risk profile are presented in chapter 4, and chapter 5 describes the efforts undertaken until now to fill these data gaps. The availability of a dose-response model for HEV infections in humans is discussed in chapter 6, followed by a general discussion and proposed future work in chapter 7.

2 Risk profile

2.1 Hepatitis E virus

2.1.1 Genome

Hepatitis E virus (HEV) was identified for the first time in 1990 (Reyes et al., 1990) and has since been a public health concern to both developing and developed countries (Purcell and Emerson, 2008). HEV consists of a non-enveloped, positive-sense, single-stranded RNA virus of approximately 7.2 kilobases and the genome contains three open reading frames (ORFs) (Figure 1) (Purcell and Emerson, 2001). The ORF1 translates into a polyprotein that comprises between 1691 and 1708 amino acids, depending on the isolate (Schlauder, 2004). The partial proteins of ORF1 are translated from sequences homologous to those that code for amongst others methyltransferase and RNA-dependent RNA polymerase, important proteins for successful replication of the virus (Purcell and Emerson, 2001). ORF2 encodes for the protein of the viral protein shell (capsid), which comprises 659 or 660 amino acids, depending on the isolate (Schlauder, 2004). The ORF3 protein is the least conservative and comprises 122 or 123 amino acids, depending on the isolate (Schlauder, 2004). The function of the ORF3 protein needs additional investigation, but the protein is suggested to be involved in intracellular immunosuppression (Schlauder, 2004; Tyagi et al., 2004).
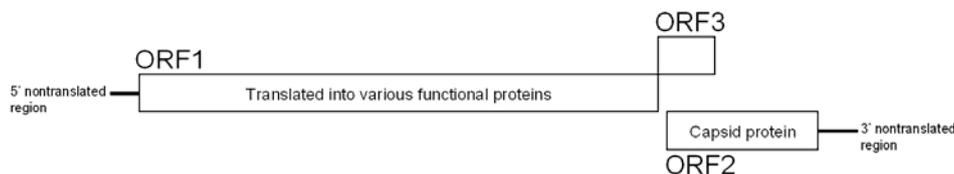
Figure 1. Genome organization of hepatitis E virus.

2.1.2 Classification

Hepatitis E virus was classified originally as a member of the *Caliciviridae* family. Based on deviations from the genomic organization of other Caliciviruses, however, HEV was reclassified recently as the sole member of the genus *Hepevirus* of the family *Hepeviridae* (Emerson et al., 2004a). Hepatitis E viruses are classified into four genotypes, consecutively named 1 through 4 (Panda et al., 2007). A possible fifth genotype is proposed for a virus in poultry that shares about 50-60% nucleotide similarity to HEV sequences of genotypes 1-4 (Haqshenas et al., 2001). A subdivision of the genotypes classifies HEV strains into five subtypes within genotype 1 (1a – 1e), two subtypes within genotype 2 (2a, 2b), 10 subtypes within genotype 3 (3a – 3j) and seven subtypes within genotype 4 (4a – 4g) (Lu et al., 2006). Based on these numbers of subtypes, HEV strains within genotype 1 and genotype 2 appear to be more conserved than HEV strains from genotype 3 and genotype 4.
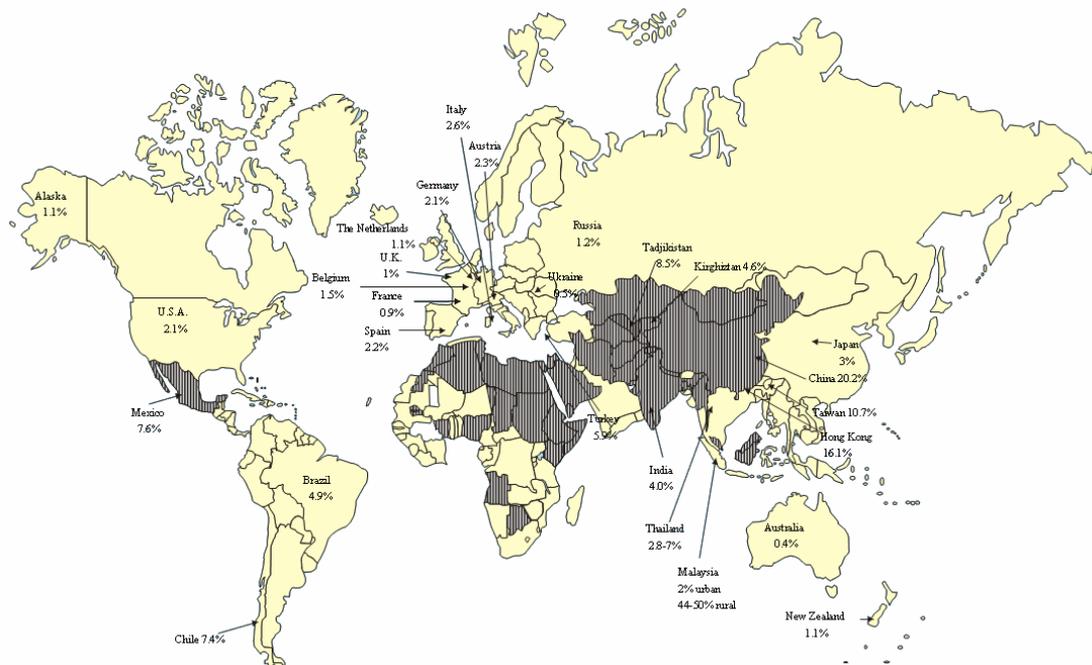
Figure 2. Global seroprevalence of anti-HEV antibodies (adapted from Worm et al., 2002). Shaded areas indicate that >25% of sporadic non-ABC cases are caused by HEV.

2.1.3 Geographical distribution

The different HEV genotypes show a distinct geographical distribution (Lu et al., 2006). Hepatitis E virus strains of genotype 1 are predominantly isolated from hepatitis E patients in Asian and African countries, both from sporadic cases and from outbreak-cases. Genotype 2 HEV strains have been observed during outbreaks in Mexico, Nigeria and Chad. Genotype 3 HEV strains are commonly associated with locally acquired hepatitis E cases in North-America, Europe, Japan and China. Genotype 4 strains of HEV are observed mostly in sporadic cases of hepatitis E in developed countries[1] in Asia, such as Japan and Taiwan, but also in developing countries such as Indonesia, China and Vietnam.

2.2 Hepatitis E virus in humans

2.2.1 Hepatitis E

Human infections by HEV can lead to clinical disease, referred to as hepatitis E. Clinical symptoms of hepatitis E in humans cannot be distinguished from the symptoms of other forms of viral hepatitis. Serologic or molecular evidence is required for the confirmation of a HEV infection as possible cause of the clinical symptoms. The general symptoms of hepatitis are anorexia, jaundice and liver

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[1] The definition of developed and developing countries used in this report is adopted from the Development Assistance Committee of the Organization for Economic Co-operation and Development (www.oecd.org/dac/stats/daclist, accessed March 16th 2009)

enlargement (Purcell and Emerson, 2001). Furthermore, about half the patients with hepatitis E display abdominal pain and tenderness, nausea and fever. Hepatitis E is mostly self-limiting and in general does not progress to chronicity (Jameel, 1999; Purcell and Emerson, 2001), although several chronic cases have been reported recently (Gerolami et al., 2008; Haagsma et al., 2008; Kamar et al., 2008). Mortality rates among patients are generally <0.5%, but may reach up to 25% in pregnant women for at least genotype 1 (Kumar et al., 2004). The reasons for the high mortality rate in pregnant women are still unknown.

2.2.2 HEV epidemiology

HEV is associated with large outbreaks of hepatitis E among humans in developing countries. Predominantly inhabitants from Asian and African countries are exposed to the virus due to poor sanitary conditions (Purcell and Emerson, 2001). Sewage overflow that results from heavy rainfall may contaminate surface water that is used for drinking water production or as source for water used for household tasks. As water is widely distributed and used, the number of people exposed is generally large, explaining the large-scale outbreaks of HEV in developing countries (first desribed by Viswanathan, 1957).

Despite the observed outbreaks in developing countries only, anti-HEV antibodies have been observed globally, including in developed countries (Figure 2). These presumed HEV infections in developed countries were initially attributed to travel to HEV endemic areas, until several serologically confirmed cases in developed countries could not be attributed to travel (Zaaijer et al., 1993; Zanetti and Dawson, 1994). In 1998, the first HEV-sequence from a locally-acquired hepatitis E patient was obtained in the USA (Kwo et al., 1997), followed by reports of locally-acquired hepatitis E cases–confirmed by HEV RNA detection in serum–in Taiwan, Greece, Italy, Spain, Japan, the Netherlands, the UK and Germany (Hsieh et al., 1998; Schlauder et al., 1999; Zanetti et al., 1999; Pina et al., 2000; Takahashi et al., 2002; Widdowson et al., 2003; Banks et al., 2004a; Preiss et al., 2006). Hence, HEV infections are also acquired locally in developed countries.

A number of epidemiological studies have focused on potential risk factors for HEV infection by analyzing characteristics of hepatitis E patients that requested medical consultation in hospitals. Ijaz et al. (2005) compared data from non-travel and travel associated hepatitis E in UK-patients and observed an increased risk for the non-travel associated form for males and for living near the coast or estuaries. Furthermore, all patients with locally-acquired hepatitis E were over 50 years of age, with the majority being over 65 years of age. Dalton et al. (2007) and Mizuo et al. (2005) both described a higher prevalence for males over females and middle-aged or elderly patients. Mizuo et al. (2005) reported that the majority of cases had consumed un(der)cooked pig liver 1-2 months before onset of hepatitis E and suffered from pre-existing diseases. Borgen et al. (2008) retrospectively interviewed 19 Dutch patients with locally-acquired hepatitis E and again found the preponderance of males over females. Furthermore, patients had a median age of 50 years and about half the patients suffered from pre-existing diseases. A case-control study on patients who acquired hepatitis E in Germany identified consumption of offal and wild boar meat as potential risk factors (Wichmann et al., 2008). Thus, recurring potential risk factors were especially gender and age, and possibly pre-existing diseases. Furthermore, two factors associated with foodborne transmission were identified. These potential risk factors should however be considered as risk factors for a severe form of hepatitis E that requires medical consultation (which relates to the design of the studies), and not per se as general risk factors for the population. Potential risk factors for the general population are contact exposure to pigs for farm workers (Hsieh et al., 1999; Drobeniuc et al., 2001), for veterinarians (Meng et al., 2002; Withers et al.,

2002; Bouwknegt et al., 2008a), for slaughterhouse personnel (Perez-Gracia et al., 2007) and for people having a pet pig (Renou et al., 2007). Another potential risk factor for the general population is consumption of uncooked dear meat (Tei et al., 2004). The importance of each of these potential routes for acquiring HEV infections in the Netherlands, however, is unknown.

Thus, risk factors for exposure to HEV are difficult to study considering the self-limiting or mild nature of infection in most cases, resulting in low numbers of identified cases. HEV has been detected in various animal species, including domestic pigs and wild boar (Goens and Perdue, 2004), and these animal HEV strains can show high similarity to human HEV strains (Meng et al., 1997; Van der Poel et al., 2001). Therefore, HEV is suggested to be a possible zoonotic virus, and human HEV exposure may involve direct or indirect contact with HEV infected animals.

2.3 Potential HEV sources

2.3.1 Animals

2.3.1.1 Domestic pigs

Balayan et al. (1990) reported the possibility of HEV infection in pigs by experimentally infecting pigs intravenously (*iv*) with HEV obtained from a human patient (the HEV genotype was unknown). The aim of the experiment was to assess whether or not HEV can replicate in vertebrates other than primates, which had been described at that time (Balayan et al., 1983; Bradley et al., 1987). Although later reports suggested that the experimentally infected pigs were already infected by HEV of genotype 3 (Lu et al., 2004), HEV was shown for the first time to be able to infect pigs. In 1995, Clayson et al. (1995) observed HEV in domestic pigs in Nepal, and raised concerns about zoonotic transmission of HEV in developed countries. In 1997, Meng et al. (1997) showed that HEV was also prevalent among domestic pigs in the USA, a non-HEV-endemic country. In addition, porcine HEV isolates from the USA were characterized genetically, showing >90% similarity between human and porcine HEV strains from the USA, corroborating the zoonosis hypothesis. These two reports catalyzed publication of reports on HEV in domestic pigs from other countries (Tables 1 and 2). Interestingly, pigs are reported to be infected by genotype 3 and genotype 4 HEV strains only, also in countries where genotype 1 prevails among humans (Arankalle et al., 2002; Cooper et al., 2005; Zheng et al., 2006). The infection of pigs by Balayan et al. (1990) was caused by an uncharacterized HEV strain that presumably belonged to genotype 1. Meng et al. (1998a) inoculated pigs *iv* with HEV genotype 1 in a later experiment, but pigs remained uninfected. Therefore, pigs may not be susceptible to HEV genotype 1.

As listed in Tables 1 and 2, HEV is ubiquitous in pigs worldwide and prevalence estimates for pig farms may reach up to 100%. These findings suggest that HEV is transmitted among pigs, and pigs were indeed shown to have the potential to transmit HEV sufficiently to explain HEV epidemics (Bouwknegt et al., 2008b). Therefore, pigs have the potential to be a true animal reservoir for HEV and are able to maintain HEV infection. Given the continuous addition of HEV-susceptible pigs in the Dutch pig fattening industry (a production round lasts about six months), HEV is likely able to persist among pigs.

Porcine HEV strains of genotype 3 can cause infection in primates and human HEV strains of genotype 3 can cause infection in pigs (Meng et al., 1998b). These data further suggest the possibility of zoonotic

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Table 1. Prevalence and identity of HEV RNA in pigs globally.

Country	Year of sampling	Sample type	No. of pigs	% pigs pos.	No. of farms	% farms pos.	No. of sequences	Genotype[1]	Ref.
Asia									
China	n.a.[2]	Serum	263	1.9	n.a.	n.a.	5	4	(Wang et al., 2002)
	2002-2004	Faeces	282	9.6	n.a.	n.a.	} 10	4	(Zheng et al., 2006)
	2002-2004	Bile	160	3.1	n.a.	n.a.			(Zheng et al., 2006)
India	2000	Serum	284	4.6	n.a.	n.a.	12	4	(Arankalle et al., 2002)
	1985-1987	Serum	45	4.4	n.a.	n.a.	2	4	(Arankalle et al., 2003)
	1999	Serum	12	33.3	n.a.	n.a.	4	4	(Arankalle et al., 2003)
	n.a.	Faeces (slaughter)	210	0.5	n.a.	n.a.	1	4	(Shukla et al., 2007)
	n.a.	Faeces	94	0	1	-	-	-	(Shukla et al., 2007)
Indonesia	2003	Serum	99	1.0	8	12.5	1	4	(Wibawa et al., 2004)
	2004	Serum	101	5.0	n.a.	n.a.	5	4	(Wibawa et al., 2007)
Japan	n.a.	Faeces	386	22.3	3	100	26	3	(Nakai et al., 2006)
	n.a.	Faeces	186	1.6	12	25	3	3	(Okamoto et al., 2001)
	2000-2002	Serum	1360	13.7	25	88	137	3, 4	(Takahashi et al., 2003)
	2001-2002	Serum	1425	3.9	92	34	55	3, 4	(Takahashi et al., 2005)
	2002-2004	Serum	152	13.8	3	66.7	22	3	(Tanaka et al., 2004)
Korea	n.a.	Serum	128	2.3	10	n.a.	3	3	(Choi et al., 2003)
	1995-2004	Hepatic tissue	388	10.8	388	10.8	42	3	(Jung et al., 2007)
Mongolia	2006	Serum	243	36.6	4	100	89	3	(Lorenzo et al., 2007)
Taiwan	n.a.	Serum	56	1.8	2	50	1	4	(Hsieh et al., 1999)
	n.a.	Serum	235	1.3	n.a.	n.a.	3	4	(Wu et al., 2000)
	1998-2000	Serum	521	1.5	n.a.	n.a.	} 4	3, 4	(Wu et al., 2002)
	1998-2000	Faeces	54	5.6	n.a.	n.a.			(Wu et al., 2002)
Thailand	n.a.	Serum	76	13.2	4	25	10	3	(Cooper et al., 2005)
Oceania									
New Zealand	n.a.	Faeces	45	37.8	2	n.a.	7	3	(Garkavenko et al., 2001)

[1] no number between brackets indicates all sequences belong to the respective genotype; [2] n.a.: not available; [3] faeces, serum, bile and hepatic tissue

Table 1. Continued.

Country	Year of sampling	Sample type	No. of pigs	% pigs pos.	No. of farms	% farms pos.	No. of sequences	Genotype (%)[1]	Ref.
Europe									
Netherlands	1998-1999	Faeces (pooled)	n.a.	n.a.	115	21.7	14	3	(Van der Poel et al., 2001)
	2005	Faeces (pooled)	n.a.	n.a.	97	54.6	38	3	(Rutjes et al., 2007)
Spain	2003-2004	Various[3]	69	37.7	23	n.a.	26	3	(De Deus et al., 2007)
	2002-2004	Faeces	146	23.3	21	38	9	3	(Fernandez Barredo et al., 2006)
	2002-2004	Faeces (pooled)	n.a.	n.a.	16	50	n.a.	n.a.	(Fernandez Barredo et al., 2006)
	>2001	Faeces	41	17.1	1	-	n.a.	3	(Seminati et al., 2008)
	>2001	Serum	66	27.3	1	-			(Seminati et al., 2008)
UK	n.a.	Faeces	40	22.5	1	-	2	3	(Banks et al., 2004b)
North America									
USA	n.a.[2]	Faeces	80	40.0	29	65.5	⎰ 27	3	(Huang et al., 2002a)
	n.a.	Serum	16	12.5	8	12.5	⎱		(Huang et al., 2002a)
	2002	Faeces (pooled)	n.a.	n.a.	28	25	7	3	(Kasorndorkbua et al., 2005)
South America									
Argentina	n.a.	Faeces	54	88.9	1	-	7	3	(Munné et al., 2006)
Mexico	n.a.	Serum	125	6.4	10	30	7	3	(Cooper et al., 2005)
	n.a.	Faeces	90	31.1	9	56	21	3	(Cooper et al., 2005)
Oceania									
New Zealand	n.a.	Faeces	45	37.8	2	n.a.	7	3	(Garkavenko et al., 2001)

[1] no number between brackets indicates all sequences belong to the respective genotype; [2] n.a.: not available

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Table 2. Prevalence of anti-HEV antibodies in sera from pigs globally.

Country	Year of sampling	Type of antibody	Number of samples	% positive samples	Number of farms	% positive farms	Ref.
Asia							
China	n.a.[1]	IgG	82	26.8	4	75	(Meng et al., 1999)
	n.a.	IgG	419	78.8	n.a.	n.a.	(Wang et al., 2002)
India	1985-1987	IgG	45	93.3	n.a.	n.a.	(Arankalle et al., 2003)
	1988	IgG	137	74.4	n.a.	n.a.	(Arankalle et al., 2001)
	1993	IgG	97	54.6	n.a.	n.a.	(Arankalle et al., 2001)
	1999	IgG	12	100	n.a.	n.a.	(Arankalle et al., 2003)
	2000	IgG	284	42.9	n.a.	n.a.	(Arankalle et al., 2002)
Indonesia	2003	IgG	99	71.7	8	100	(Wibawa et al., 2004)
Japan	.	IgG	107	39.3	3	100	(Nakai et al., 2006)
	2000-2002	IgG	2500	57.9	25	100	(Takahashi et al., 2003)
	2001-2002	IgG	1425	55.7	n.a.	n.a.	(Takahashi et al., 2005)
	2001-2002	IgM	1425	7.0	n.a.	n.a.	(Takahashi et al., 2005)
	2001-2002	IgA	1425	11.7	n.a.	n.a.	(Takahashi et al., 2005)
	2002-2004	IgG	152	13.0	n.a.	n.a.	(Tanaka et al., 2004)
Korea	n.a.	IgG	264	14.8	13	85	(Choi et al., 2003)
	n.a.	IgG	140	40.7	n.a.	n.a.	(Meng et al., 1999)
Lao	1998	IgG	301	15.3	n.a.	46	(Blacksell et al., 2007)
	2001	IgG	586	51.2	n.a.	n.a.	(Blacksell et al., 2007)
Mongolia	2006	IgG	243	91.8	4	100	(Lorenzo et al., 2007)
Taiwan	n.a.	IgG	275	37.1	10	90	(Hsieh et al., 1999)
	1998-2000		521		n.a.	n.a.	(Wu et al., 2002)
Thailand	n.a.	IgG	75	30.7	4	75	(Meng et al., 1999)

[1] n.a.: not available

Table 2. Continued.

Country	Year of sampling	Type of antibody	Number of samples	% positive samples	Number of farms	% positive farms	Ref.
Oceania							
Australia	n.a.	IgG	131	20.6	4	75	(Chandler et al., 1999)
New Zealand	n.a.	IgG	72	75.0	22	91	(Garkavenko et al., 2001)
Europe							
Netherlands	n.a.[1]	IgG	34	23.5	n.a.	n.a.	(Banks et al., 2004b)
Spain	1998-2000	IgG	439	41.9	41	98	(Seminati et al., 2008)
	1998-2000	IgM	418	28.2	41	83	(Seminati et al., 2008)
Sweden	n.a.	IgG	204	58.0	n.a.	n.a.	(Banks et al., 2004b)
UK	1991-2001	IgG	256	85.5	n.a.	n.a.	(Banks et al., 2004b)
North America							
Canada	n.a.	IgG	712	18.1	67	55	(Meng et al., 1999)
Canada	1998-2000	IgG	998	59.5	n.a.	n.a.	(Yoo et al., 2001)
USA	n.a.	IgG	84	34.5	4	100	(Withers et al., 2002)
USA	n.a.	IgG	283	71.4	15	100	(Meng et al., 1997)
South America							
Argentina	n.a.	IgG	97	22.7	5	100	(Munné et al., 2006)
Brazil	n.a.	IgG	427	57.1	n.a.	n.a.	(Vitral et al., 2005)
Mexico	n.a.	IgG	125	80.0	10	30	(Cooper et al., 2005)

[1] n.a.: not available

transmission of genotype 3 swine-HEV. To date, no direct association between pigs and human HEV infection has been reported. Several epidemiological studies, however, related direct contact with pigs to exposure to HEV, as an elevated HEV seroprevalence was observed in swine farm workers and veterinarians compared to the respective control groups (Hsieh et al., 1999; Drobeniuc et al., 2001; Meng et al., 2002; Withers et al., 2002). Also foodborne transmission of swine HEV is proposed, because porcine liver from retail may contain HEV RNA (Yazaki et al., 2003) and infectious HEV (Feagins et al., 2007). HEV likely can cause infection in humans after oral ingestion, as several Japanese persons developed hepatitis E after consumption of uncooked deer meat and identical HEV strains were obtained from patients and the meat (Tei et al., 2003).

Pigs can become infected and excrete HEV faecally at the age of two weeks (Fernandez Barredo et al., 2006; LeBlanc et al., 2007). The highest prevalence of faecal HEV excretion is observed among pigs from 10 weeks of age until 3 months (Nakai et al., 2006; LeBlanc et al., 2007; Seminati et al., 2008). This finding might indicate that HEV infection occurs soon after the onset of fattening, which lasts from about 10 weeks of age until slaughter at about 26 weeks of age. Pigs are reported to become viremic on average at 2-3 months of age and seroconversion (*i.e.,* first detection of antibodies) to HEV is observed generally between 2-4 months of age (Takahashi et al., 2003; Takahashi et al., 2005).

2.3.1.2 Poultry

A virus that showed a similar genomic organization and significant sequence identity with HEV was found in chickens with hepatitis-splenomegaly in 2001 (Haqshenas et al., 2001). This virus, designated avian HEV, is genetically related but distinct from human and swine HEV. The genome is about 600 nucleotides shorter than that of human and swine HEV (Huang et al., 2004). In the USA, about 70% of 76 chicken flocks raised at least one anti-avian HEV antibody positive chicken and about 30% of 1,276 individual chickens tested positive by ELISA (Huang et al., 2002b). No avian HEV has been reported in the Netherlands.

Avian HEV is infectious to both chicken and turkey (Sun et al., 2004), but rhesus monkeys inoculated intravenously remained uninfected (Huang et al., 2004). Considering swine HEV is infectious to rhesus monkeys and therefore assumed to be potentially pathogenic to humans (Meng et al., 1998b), avian HEV may not pose a public health concern. This hypothesis is corroborated by the absence of avian HEV sequences among human patients. However, further research is required, because the observed cases are usually those that require hospitalization and therefore are the severe hepatitis E cases.

2.3.1.3 Cattle

Anti-HEV IgG was found in 6% (n=290) of cattle in China (Wang et al., 2002; Zhang et al., 2008a), in 1% (n=70) of cattle in Brazil (Vitral et al., 2005) and in 6% (n=279) of cattle in India (Arankalle et al., 2001). Neither HEV RNA isolation, nor inoculation of cattle with HEV have been reported to date. Furthermore, no research has been reported on HEV detection in cattle in other countries, including the Netherlands. Therefore, it is currently unknown whether or not cattle are a potential HEV reservoir.

2.3.1.4 Horses

Anti-HEV IgG was detected in 13% of 200 horses in Egypt and in 16% of 49 horses in China (Saad et al., 2007; Zhang et al., 2008a). Furthermore, the same studies report isolation of HEV RNA in four horses in Egypt (all HEV of genotype 1) and a single horse in China (HEV of genotype 3), respectively. These findings suggest that horses may potentially be a HEV reservoir. However, no data are reported for HEV prevalence among horses in other countries, including the Netherlands.

2.3.1.5 Sheep

Anti-HEV IgG was detected by ELISA in all of 58 examined samples from sheep in India, but the specificity of the reactivity could not be confirmed by inhibition studies for any of the samples (Shukla et al., 2007). Furthermore, no anti-HEV IgG was detected in 12 sheep samples from Brazil (Vitral et al., 2005). Samples from sheep in other countries have not been reported to be examined, including the Netherlands, nor were efforts to detect HEV RNA from samples from sheep.

2.3.1.6 Goats

Anti-HEV IgG was detected by ELISA and confirmed by inhibition assays in 12 out of 50 goat samples in China (Zhang et al., 2008a) and in 86 out of 86 goat samples in India (Shukla et al., 2007). In contrast, none of 316 goat samples from China (Wang et al., 2002), none of 250 goat samples from India (Arankalle et al., 2001) and none of five goat samples from Brazil (Vitral et al., 2005) showed reactivity. Goats could not be infected by HEV of genotype 1 after intravenous inoculation (Arankalle et al., 2001), but no inoculation attempts were made with HEV of genotype 3. Lambs were reported to be infected after inoculation by strains Osh-225 and Osh-228 (obtained from humans) (Usmanov et al., 1994), but this report is in Russian hampering accurate interpretation of results. Inoculation of lambs is not reported to have been repeated by other research groups.

2.3.1.7 Cats

Anti-HEV IgG was detected by ELISA and confirmed by inhibition assays in 4 out of 202 (Mochizuki et al., 2006) and in 22 out of 135 (Usui et al., 2004) serum samples from cats in Japan. Furthermore, a stray cat kept as pet by a Japanese hepatitis E patient was serologically positive for anti-HEV antibodies (Kuno et al., 2003). Whether the anti-HEV seropositivity represents former or current HEV infections, and/or false-positive test results is unknown. Furthermore, the association between the human HEV infection and the presumed feline HEV infection is unclear. In Egypt, frequent contact with cats was identified as potential risk factor for anti-IgG reactivity in serum of pregnant women (Stoszek et al., 2006), suggesting a possible role of domestic cats in exposure of humans to HEV. No data on HEV RNA isolation from cats has been reported to date, thus it is unknown whether cats are a host for HEV.

2.3.1.8 Dogs

Anti-HEV IgG was detected by ELISA and confirmed by inhibition assays in 21 out of 101 dogs in China (Zhang et al., 2008a), in 10 of 22 dogs in India (Arankalle et al., 2001), in 3 of 43 dogs in Brazil (Vitral et al., 2005). Whether the anti-HEV seropositivity represents former or current HEV infections, and/or false-positive test results is unknown. Furthermore, the association between the human HEV infection and the presumed canine HEV infection is unclear. No data on HEV RNA isolation from dogs has been reported to date.

2.3.1.9 Wild boar

In several Japanese studies wild boar have been analyzed by serology or molecular methods for the presence of HEV-specific antibodies or HEV RNA. Seroprevalences were observed that varied from 9% (Sonoda et al., 2004) to 25% (Chandler et al., 1999; Michitaka et al., 2007). Prevalences of HEV RNA in sera and/or liver varied from 2% to 43%, whereby the high prevalence of 43% was based on a low number (n=7) of animals (Takahashi et al., 2004; Nishizawa et al., 2005; Michitaka et al., 2007). Michitaka et al. (2007) studied wild-caught boar as well as boar kept in a breeding farm. They demonstrated that the seroprevalence in the bred boar (71.4%) was significantly higher than in the wild-caught boar (25.5%). Furthermore, they showed that wild isolates obtained from wild boar hunted in the same habitat formed a phylogenetic cluster, while other independent isolates were from different

regions. In Europe, HEV RNA positive wild boar samples have been found in Italy, Spain and Germany. The lowest prevalence was found in Germany, where in 5.3% of 189 sera HEV RNA was detected by RT-PCR (Kaci et al., 2008). In Spain, 19.6% of 138 sera were positive for HEV RNA (de Deus et al., 2008) and in Italy, 25% of 88 bile samples from wild boar were found to be positive for HEV RNA (Martelli et al., 2008).

2.3.1.10 Deer

Also several species of deer have been studied for the presence of HEV-specific antibodies and HEV RNA. In Japan, 2% of the Sika deer (*Cervus nippon*) were shown to be seropositive for HEV (Sonoda et al., 2004; Matsuura et al., 2007) and HEV RNA has been isolated from these deer as well (Tei et al., 2003; Takahashi et al., 2004). In Eastern China, in 2 of 8 Sika deer and in 4 of 8 tufted deer (*Elaphodus cephalophus*) HEV RNA was detected (Zhang et al., 2008b). In the USA, in none of 174 Sika deer samples HEV-specific antibodies were detected, suggesting that Sika deer in those USA populations, unlike those in Japan and China, are not a source of HEV (Yu et al., 2007). This contrast with Japan and China may be explained by the fact that Sika deer in those countries live in close proximity to swine and wild boar populations that are known to harbor HEV.

2.3.1.11 Mongooses

Anti-HEV antibodies have been detected in serum of 21 out of 100 (21%) mongooses collected in Okinawa (Japan) in 2002 (Nakamura et al., 2006) and in 7 out of 84 (8%) serum samples from mongooses collected in 2004-2005, also in Okinawa (Li et al., 2006a). The ELISAs used were in-house ELISAs based on HEV of genotype 1 (Nakamura et al., 2006) and HEV of genotype 1, 3 and 4 (Li et al., 2006a). No differences in reactivity were observed among the different antigens.

HEV RNA was detected in a single serum sample and sequence analysis characterized the variant as genotype 3 (Nakamura et al., 2006). The full-genome showed 99.5% similarity to a HEV-variant recovered from a pig in Okinawa. Mongooses live in southern Asia, southern Europe, Africa and the Caribbean and Hawaiian islands. No other reports about the detection of HEV RNA or anti-HEV antibodies in mongoose were found.

2.3.1.12 Rabbits

Out of 335 rex rabbits from two commercial farms in China, 191 (57%) tested positive for anti-HEV antibodies by double antigen sandwich enzyme immunoassay (Zhao et al., 2009). Twenty-five of the 335 rabbits (7.5%) tested positive for HEV RNA in serum by nested RT-PCR directed at ORF2, with positivity-rates of ~7% among the rabbits with and without detected anti-HEV antibodies. Two full length genomes were obtained from rabbits on one of the farms, and sequence analysis revealed 85% similarity to each other and 74%, 73%, 78-79%, 74-75% and 46-47% similarity to genotypes 1 through 5, respectively (Zhao et al., 2009). These findings suggest the existence of an additional HEV genotype in rabbits in China. No reports on HEV in rabbits from other countries were found.

2.3.1.13 Rodents

HEV-specific antibodies were found in rats (*Rattus norvegicus, R. rattus* and *R. exulans)* from India (Arankalle et al., 2001), Nepal (He et al., 2002) and the USA (Kabrane-Lazizi et al., 1999; Favorov et al., 2000; Easterbrook et al., 2007). In the USA, *Rattus norvegicus* were more frequently anti-HEV seropositive than *R. rattus* (69% versus 38%) and rodents captured in urban areas showed an approximate two-fold higher seroprevalence (60%) compared to rodents captured in rural areas (27%) (Favorov et al., 2000). In India, *R. rattus* were more frequently anti-HEV seropositive than *R. norvegicus* (10% versus 0%) (Arankalle et al., 2001). Rodents other than rats testing positive for HEV-specific antibodies were *Nectomus sp.* in Brazil (2 of 4) (Vitral et al., 2005), *Bandicota bengalensis*

(8 of 39) in Nepal (He et al., 2002) and *Neotoma sp.* (62 of 114), *Sigmodon hispidus* (36 of 110), *Peromyscus sp.* (19 of 194), *Oryzomys palustris* (10 of 41), *Clethrionomys gaperi* (4 of 6) and *Mus musculus* (2 of 14) in the USA (Favorov et al., 2000). In contrast, HEV-specific antibodies were not detected in serum samples from 58 house mice (*Mus musculus domesticus*) and three Norway rats (*R. norvegicus*) captured in or near swine-houses in the USA (Withers et al., 2002).

HEV RNA was reportedly detected in blood from Nepalese rats (He et al., 2002), suggesting virus replication and thus susceptibility of rats to HEV, but this finding was found to be related to laboratory contamination rather than infection of rats by HEV (He et al., 2006).

Experimental intravenous inoculation of Wistar rats with an unspecified volume of an inoculum containing 1.3×10^2 HEV PDU per ml reportedly resulted in infection in all of 27 rats (Maneerat et al., 1996). Faeces and blood samples were collected on every fourth day postinoculation until day 35 and HEV-shedding was observed on day 7 in three of three examined rats. Viremia was inconsistently detected in some rats during the 35-day study period. The HEV genotype infecting the rats is unknown. The inoculum was infectious to non-human primates, suggesting the presence of genotype 1, but also to pigs, suggesting the presence of HEV of genotype 3 or 4 (Maneerat et al., 1996).

2.3.1.14 Musk rats

No HEV RNA was detected in 150 faecal samples from musk rats (*Ondatra zibethicus*) collected in 1998 and 1999 in the Netherlands (Rutjes et al., 2009b).

2.3.2 Food

2.3.2.1 Pork

Porcine livers have been found to contain HEV RNA in Japan, the USA and the Netherlands (Yazaki et al., 2003; Bouwknegt et al., 2007; Feagins et al., 2007). Commercial porcine livers in the UK were tested to be negative (Banks et al., 2007). Presence of infectious HEV could not be confirmed for Dutch commercial porcine livers (Bouwknegt et al., 2007), whereas those obtained in the USA contained infectious HEV (Feagins et al., 2007). Thus, porcine liver may cause foodborne HEV transmission to humans.

Twenty of thirty-nine muscle samples that were proxies for pork meat at retail from pigs contact-infected by HEV in an experiment contained HEV RNA, suggesting possible foodborne transmission through pork meat consumption (Bouwknegt et al., 2009). Contamination of meat likely is a consequence of viremia, suggesting that meat obtained from pigs in the acute phase of infection at slaughter can be contaminated by HEV. In the Netherlands, 14% of pigs at slaughter were found to excrete HEV RNA (Rutjes et al., manuscript in preparation) and may have been viremic, suggesting a considerable portion of produced pork meat may be contaminated by HEV. For transmission through pork meat, however, infectious HEV needs to be present, which is currently not confirmed. Furthermore, the stability of HEV is unknown due to the absence of a cell culture system, hindering the estimation of the effect of for instance storage on HEV concentrations. HEV is heat inactivated (Emerson et al., 2005; Tanaka et al., 2007; Feagins et al., 2008), indicating proper cooking prior to consumption lowers the risk of foodborne transmission. It will be worthwhile to screen pork at retail for HEV RNA, to assess the HEV RNA concentration and to determine whether infectious HEV is present.

2.3.2.2 Game meat

Wild boar and deer are suspected sources of foodborne zoonotic transmission of HEV. In Japan, several cases of hepatitis E have been linked epidemiologically to eating undercooked pork liver or wild boar meat (Matsuda et al., 2003; Yazaki et al., 2003; Masuda et al., 2005). Most direct evidence of zoonotic HEV transmission was obtained when four cases of hepatitis E were linked directly to eating raw deer meat by the presence of identical HEV strains in the consumed deer meat and patients (Tei et al., 2003). Furthermore, zoonotic transmission of HEV genotype 3 from wild boar to human was demonstrated by only one nucleotide difference in a sequence of 1,980 nucleotides of the entire ORF2 genome (99.95% identity) in HEV isolated from a patient and the wild boar meat she consumed (Li et al., 2005).

2.3.2.3 Shellfish

The presence of HEV of genotype 3 has been reported for 2 of 32 packages of Yamato-Shijimi in Japan (Li et al., 2007). No other data for shellfish have been reported (new data are presented in section 5.2.2 of this report).

2.3.3 Water

2.3.3.1 Wastewater

Several studies have shown that HEV genotype 3 is consistently present in sewage water that might originate from pigs and humans (Albinana-Gimenez et al., 2006, Clemente-Casares et al. 2003, Kasorndorkbua et al., 2005, Pina et al., 2000). Animals were used to show infectivity of HEV in water since no susceptible cell line was available. Indeed rhesus monkeys subjected to HEV in Spanish sewage concentrates showed signs of infection (Pina et al., 1998). In Spain, over 40% of urban sewage samples tested positive for HEV RNA. Treatment processes may not be efficient in the reduction of numbers of these small, environmentally stable viruses (Lodder and de Roda Husman, 2005).

2.3.3.2 Surface water

For HEV, large waterborne outbreaks have been described (for instance Naik et al., 1992). Transmission of pathogenic enteric viruses such as HEV occurs by the faecal oral-route. Exposure to this virus through contaminated surface water may occur directly, through drinking or bathing, or indirectly by consumption of treated surface water or consumption of foods contaminated by surface water used for irrigation or washing. Because large surface waters such as the river Meuse receive input from both human and animal sources, discharge human viruses such as HEV may be detected as well as zoonotic viruses such as HEV. In the Netherlands, HEV RNA was shown to be present in 2 of 12 monthly water samples from the river Meuse (Rutjes et al., 2009b).

3 HEV detection

3.1.1 Recovery of HEV RNA

The estimated recovery of a detection method is the fraction of HEV presenting the sample that is extracted and detected following the RNA isolation and detection by RT-PCR. This parameter is required to be adjusted for in the risk assessment, because the actual HEV dose being exposed to is underestimated otherwise. However, estimates for the recovery yielded by the HEV detection assays are not reported to date.

3.1.2 Molecular detection

Detection of (parts of) the HEV genome with a high positive predictive value can be done by polymerase chain reaction (PCR) (Mullis et al., 1986; Erlich et al., 1988). A reverse transcription (RT) step to generate DNA is required prior to PCR amplification for RNA targets. For HEV, many different RT-PCR assays have been described currently (for instance Meng et al., 1997; Schlauder et al., 1999; Wang et al., 1999; Mizuo et al., 2002). The choice of which RT-PCR to use for HEV detection depends on the purpose of the analysis. For HEV detection, the RT-PCR with highest sensitivity is desired. For source attribution or typing of identified strains, the targeted fragment should have sufficient length and variability to increase the discriminative power for phylogenetic analysis.

HEV detection in environmental and animal samples in the Netherlands is mostly done by the conventional RT-PCR targeting a 197 base pair-fragment of ORF2 (Van der Poel et al., 2001; Widdowson et al., 2003; Waar et al., 2005; Bouwknegt et al., 2007; Rutjes et al., 2007; Rutjes et al., 2009b).

3.1.3 Typing

Genotyping of HEV strains from patients in developed countries can distinct travel-related cases from locally-acquired cases, because HEV of genotype 1 and 2 is most likely acquired in developing countries, whereas genotypes 3 is most likely acquired in developed countries. HEV of genotype 4 is most likely of Asian origin.

Furthermore, the percentages of similarity between HEV strains within a genotype is a measure of relatedness and can provide some information about the transmission between possible sources of HEV (Rutjes et al., 2009b). A 100% similarity (*i.e.,* homologous strains) strongly suggests a patient-source relation (Takahashi et al., 2004). The power of such a claim, however, depends on the size of the fragments on which the similarity is based. When the size of fragments is relatively short and the specific region targeted conserved, then homology between strains can be observed in the absence of an apparent relation (Rutjes et al., 2009b). Ideally, full genome sequences are compared, but efforts to obtain those are costly and not always possible technically (*i.e.*, for samples with low titers or with components that inhibit PCR amplification).

Sequences of ~300–450 nucleotides in the 5'-end of the ORF2 region are the most conserved among all HEV isolates and account for the majority of HEV sequences published (Lu et al., 2006). This fragment is sufficiently variable to distinct genotypes and subgroups within genotypes. The fragment of 197 base pairs of ORF2, as used mostly in the Netherlands (see section 1.4.2), is sufficiently variable to distinct genotypes, but not to distinct subgroups within genotypes. Analysis of a fragment of 287 base pairs of ORF1 obtained by a nested RT-PCR as described by Rutjes et al. (2007) yields sufficient discriminative power to enable subgrouping within genotypes.

3.1.4 Infectivity

Only infectious HEV particles can cause infection in susceptible hosts. The RT-PCR procedure detects genomes of both intact and defective virus particles. Thus, to infer on possible public health risks using RT-PCR data, the fraction of infectious HEV among the detected genomes should be known. In theory, this fraction can be assessed by quantifying HEV in samples by cell culture and RT-PCR and calculating the quotient (RT-PCR concentration as numerator), as was done for instance for enteroviruses (De Roda Husman et al., 2009). Two methods are described to assess the infectivity of HEV in samples: cell culture and use of live pigs (*i.e.,* a bioassay).

3.1.4.1 Cell culture

Successful propagation of HEV on cell culture has been reported for 2BS and LLC-MK2 cells (Huang et al., 1992), for A549 cells (Huang et al., 1995; Tanaka et al., 2007), for FRhK-4 cells that were co-cultured with primary kidney cells from a HEV infected cynomolgus monkey (Kazachkov et al., 1992), for primary hepatocytes from HEV infected macaques (Tam et al., 1996), for PLC/PLF/5 cells (Divizia et al., 1999) and for PLC/PRF/5 cells (Tanaka et al., 2007). None of these systems, however, has currently been reported to be successfully implemented by other research groups, possibly portraying the difficulty (practical or technical) of successful HEV replication in these systems.

A successful cell infection system on HepG2/C3A cells is reported for HEV that enables the study of HEV entrance within cells and thus the infectivity of HEV (Emerson et al., 2004b; Emerson et al., 2005). HEV replication, propagation to uninfected cells and cytopathic effects, however, were not observed with this system. This system is currently not reported to be in use by other research groups.

3.1.4.2 Bioassay

A bioassay assesses the infectivity of a pathogen by inoculation of the pathogen into a susceptible host, usually animals. If infection occurs, then at least some of the pathogens in the inoculum were infectious. A bioassay for HEV has been reported, using pigs as inoculated animals because pigs are readily infected by the intravenous route (Kasorndorkbua et al., 2002). This bioassay proved useful to assess the infectivity of HEV from pig manure in storage facilities and porcine livers obtained in grocery stores (Kasorndorkbua et al., 2002; Feagins et al., 2007; Feagins et al., 2008).

4 Identified data gaps

Most data gaps will arise after completion of the conceptual model and identification of parameters that are required in the risk assessment model. However, some data gaps already arise from studying literature and the current risk profile, being:
- the presence of HEV in other potential animal sources in the Netherlands other than domestic pigs, especially wild boar and deer;
- the presence of HEV in Dutch surface waters, potentially leading to exposure of humans to HEV through drinking water or water recreation;
- the potential foodborne transmission routes in the Netherlands, especially pork, game meat and shellfish.

Furthermore, it is eminent for the risk assessment to assess the presence of HEV in the Netherlands as sensitively as possible. Therefore, further improvement of the HEV detection assays as well as the statistical procedures for estimating the HEV concentration are required. The efforts undertaken up until now to fill these data gaps are described in chapter 5 of this report.

5 Data collection

5.1 Improvement of HEV diagnostics

5.1.1 Nucleic acid sequence based amplification

5.1.1.1 Introduction

Nucleic acid sequence based amplification (NASBA) is the enzymatic amplification of RNA performed under isothermal conditions. The amplification product of NASBA is single-stranded RNA. Similar to PCR, NASBA requires two primers, but the reverse primer is extended with a complementary T7-RNA polymerase promotor sequence. The first round of amplification involves attachment of the reverse primer and synthesis of a cDNA copy using AMV-RT. The enzyme RNAse H subsequently hydrolyses the RNA of this RNA/DNA hybrid and the forward primer can anneal to the remaining single stranded DNA. This substrate is again suitable for reverse transcription, rendering a double stranded DNA fragment with a functional and active T7-RNA polymerase promotor sequence. Subsequently, T7-polymerase produces multiple copies of antisense RNA transcripts. Each of these transcripts can subsequently function as template for further RNA production. The main advantage of NASBA as compared to real-time RT-PCR is the fact that NASBA is less prone to inhibition caused by inhibitory substances present in environmental samples (Rutjes et al., 2006). Moreover, analyses can be performed without a thermal cycler and because reverse transcription continues during the full amplification reaction, the effect of reverse transcription on the eventually produced number of copies may be less.

Detection of amplified RNA can be based on end-point detection of an electrochemiluminescent (ECL) signal or on real-time detection of a fluorescent signal. For ECL detection, the forward primer is complexed to a ruthenium chelate and serves as ECL probe. For real-time fluorescent detection, a stem-loop structured molecular beacon is required, which consists of a loop-sequence similar to the target sequence and two short complementary flanking sequences that forms the stem. The 5'-terminus of the probe contains a fluorophore (6-FAM or ROX) and the 3'-terminus a quencher (DABSYL). In the absence of target sequence, the stemloop-structure causes quenching of the fluorophore. In the presence of a target sequence, the loop attaches to the target sequence, causing the unfolding of the stemloop-structure. This separates the quencher and the fluorophore, resulting in a real-time detectable fluorescent signal.

5.1.1.2 Materials and methods

Primer selection

Primers targeting the 3'-end of ORF2 and the ORF2/ORF3 overlapping region of HEV were selected, and are listed in Table 3. Table 4 lists the beacons that were examined. All conditions prescribed by the manufacturer to increase the functionality of primers and beacons were acknowledged. Working solutions of primers and beacons were prepared as 20 μM and 10 μM solutions, respectively, and stored at -20 °C.

Table 3. Primers used in the NASBA for HEV RNA.

Primer	Sequence (5' → 3')	Location[1]	Based on
TaqHEV-F	GGCCGGYCAGCCGTCTGG	5179-5196	Enouf *et al.* (2006)
LCHEV-s1	TTYTGCCTATGCTGCCCGCGCCA	5154-5176	-
Forward-3	GGTGGTTTCTGGGGTGA	5231-5248	Jothikumar (2006)
HEVORF2con-s1a	GACAGAATTRATTTCGTCGGCTGG	6270-6293	Schlauder *et al.* (1999)
HEVORF2con-s1b	GACAGAATTRATTTCGTCGGCYGG	6270-6293	Manuscript in prep.
TaqHEV-R[2]	GCGAAGGGGTTGGTTGGATGA	5284-5304	Enouf *et al.* (2006)
LCHEV-a1[2,3]	AGAGGAAGGGGTTGGTTGGATGAA	5284-5300	Enouf *et al.* (2006)
HEVORF2con-a1[2]	CTTGTTCRTGYTGGTTRTCATAATC	6442-6466	Schlauder *et al.* (1999)

[1] SAR-55 (GenBank: M80581) served as reference

[2] the sequence is preceded by the T7 promotor sequence AATTCTAATACGACTCACTATAGG

[3] the short sequence AGAGGA was added to the primer sequence, because a C or T in the first 10 nucleotides after the T7 promotor sequence may abort transcription.

Table 4. Beacons used in the NASBA for HEV RNA.

Primer	Sequence (5' → 3')	Location[1]	ΔG[2]	Signal ratio[3]
Beacon-1 (B1)	CGATCGGTTGATTCTCAGCCCTTCGCGATCG	5252-5270	-2.77	9[4]
Beacon-2 (B2)	CGATCGGGTGGTTTCTGGGGTGACGATCG	5231-5247	-2.89	18
Beacon-3 (B3)	CGATCGGAGAATGCICAGCAGGACAAGGGCGATCG	6367-6389	-2.36[5]	36
Beacon-4 (B4)	CGATCGGAGAATGCICAGCAGGATAAGGGCGATCG	6367-6389	-2.36[5]	35

[1] HEV strain SAR-55 (GenBANK: M80581) served as reference

[2] free energy in kcal/mole. This value should be around -3 ± -0.5 kcal/mole

[3] Measured fluorescence in open formation divided by the measured fluorescence in closed formation. A value >2 is considered to be sufficient for detection of amplification.

[4] The ratio was re-examined three weeks later (storage at -20°C) to be 6

[5] Two possible closed formations exist. The second formation had a ΔG equal to -1.97

NASBA protocol

NASBA reactions were performed with the NucliSens Basic kit (bioMérieux, Boxtel, the Netherlands) according to the instructions of the manufacturer. Five microliters of RNA or 10-fold dilutions of this RNA, 80 mM KCl (unless stated otherwise), final primer concentrations of 0.2 μM, and final molecular beacon concentrations of 0.1 μM in a total volume of 15 μl were incubated at 65 °C for 2 min, followed by incubation at 41 °C for 2 min. Five μl of enzyme mix from the kit was added, followed by a short centrifugation step. Reagents were mixed by tapping the tubes and briefly centrifuged again. Next, real-time detection of NASBA amplicons was done for 2 h at 41 °C using a NucliSens EasyQ analyzer (bioMérieux).

5.1.1.3 Results and discussion

Primer and beacon combinations

Different combinations of primers and beacons were evaluated (Table 5). None of the examined primer and probe combinations resulted in a detectable amplification signal, despite the presence of HEV RNA in the analyzed samples as was confirmed by conventional RT-PCR. These findings may indicate that 1) the primers can not anneal properly to the target, 2) the beacons can not anneal properly to the target, 3) the T7-RNA polymerase promotor does not function properly, 4) the concentration of KCl or pH is

Table 5. Overview of the primer and beacon combinations that were examined to develop a real-time NASBA for HEV.

Forward primer	Reverse primer		
	TaqHEV-R	LCHEV-a1	HEVORF2con-a1
TaqHEV-F	Beacon-1, Beacon-2	Beacon-1, Beacon-2	-
LCHEV-s1	Beacon-1, Beacon-2	Beacon-1, Beacon-2	-
Forward-3	Beacon-1	Beacon-1	-
HEVORF2con-s1a	-	-	Beacon-3, Beacon-4
HEVORF2con-s1b	-	-	Beacon-3, Beacon-4

Table 6. Tests that were conducted to develop a real-time NASBA for HEV.

Aspect evaluated	Used oligonucleotide			mM KCl	HEV strain[1]	Matrix
	Forward	Reverse	Beacon			
Primer attachment on DNA	F1	R1	B1, B2	80	3c and 3f	faeces
KCl concentration	F1	R1	B1, B2	50 – 110	3c and 3f	faeces
pH of mixture	F2	R1, R2	B2	80	3c and 3f	faeces
Conv. PCR on NASBA products	F1, F2	R1, R2	B1	80	3c	faeces
NASBA on conv. RT-PCR products	F2	R1, R2	B1, B2	80	3c and 3f	faeces
Inhibition	F2	R2	B2	80	3c, 3f, 3k[2]	liver, bile, faeces
RNA extraction methods	F2	R2	B2	80	3c, 3f, 3k	faeces, serum
High HEV-titer	F2	R2	B2		3k	liver, bile

F1: TaqHEV-F; F2: LCHEV-s1; R1: TaqHEV-R; R2: LCHEV-a1; B1: Beacon-1; B2: Beacon-2.
[1] subtyping based on Lu et al. (2006)
[2] provisionally designated 3k, but further characterization is required.

restricting the amplification, or 5) amplification is inhibited by components co-extracted during RNA extraction.

Primer annealing

To examine whether primers were able to anneal to the target RNA, a conventional RT-PCR was done with the NASBA primers (Table 6). Fragments of the expected size (122 or 126 base pairs, depending on the primer combinations) were observed after gel electrophoresis, suggesting the primers had the correct sequence to anneal. However, sequence analysis of the PCR fragments did not produce data to confirm the specificity of the fragments.

Moreover, secondary and tertiary structures that may be present in the RNA may prevent primer annealing after denaturation of the RNA at the relatively low temperature of 65 $^{\circ}$C. Two samples were therefore heated at 95 $^{\circ}$C for 2 min prior to NASBA to examine the potential effect of denaturation. No amplification was observed after this additional denaturation step. From these experiments it remains unclear why no NASBA products were detected.

Beacon annealing

To study an alternative method for detection by molecular beacons, the presence of possible NASBA products was examined by doing a conventional PCR on the NASBA products. If cDNA and/or

dsDNA was formed during NASBA, then this cDNA/dsDNA should be detectable by PCR. The PCR reagents mixture contained 1.25 mM MgCl$_2$, 0.2 mM dNTPs, and 20 μM forward (TaqHEV-F and LCHEV-s1) and reverse (TaqHEV-R and LCHEV-a1) primer in a total volume of 45 μl. Subsequently, 5 μl of the NASBA samples was added. PCR conditions were: 3 min denaturation at 94 ^{o}C, 40 cycles of 1 min at 94 ^{o}C, 1.5 min at 60 ^{o}C and 0.5 min at 74 ^{o}C, and 7 min elongation at 74 ^{o}C. Potentially specific PCR fragments were excised form the gel, purified using the QiaQuick gel extraction kit (Qiagen) and subjected to sequence analysis. PCR fragments were observed after gel electrophoresis, but the specificity of the fragments could not be determined based on size. Fragments were excised from the gel, purified with the QiaQuick gel extraction kit (Qiagen) and subjected to sequencing. Sequencing, however, did not produce data to confirm the specificity of the fragments. Thus, it remains unclear whether the absence of detectable NASBA amplification products is caused by a defect in amplification or detection.

Functionality of the T7- RNA polymerase promotor

RT-PCR amplification products obtained by conventional RT-PCR using the NASBA primers (see 1) are double-stranded DNA molecules with a T7-RNA polymerase promotor sequence incorporated. These RT-PCR fragments were excised after gel electrophoresis, purified with the QiaQuick Gel extraction kit (Qiagen, Venlo, the Netherlands) and examined by NASBA, to study whether a functional T7-RNA polymerase promoter was present in the RT-PCR products. Again, no amplification signal was observed, suggesting that 1) the excised PCR fragments were nonspecific, 2) that the T7-RNA polymerase promotor locus does not function properly or 3) that the beacons do not anneal to the target properly.

KCl concentration and pH

The effect of different KCl concentrations was examined by using NASBA mixtures with 50, 70, 90 and 110 mM KCl. Again, no amplification signal was observed for any of the mixtures, leaving failure of amplification to be explained by the other possible reasons mentioned previously.
The effect of pH was examined by replacing 0.5 μl, 1 μl and 1.5 μl of water in the NASBA mixture by an equal volume of Tris-EDTA buffer of pH=9.5. No amplification signal was observed, suggesting the pH was not the direct cause for the amplification failure.

Inhibition

The RNA extraction methods can differ on RNA yield and the amount of co-extracted inhibitors or residual reagents, which may affect amplification. Therefore, RNAs obtained by three different extraction methods (magnetic silica beads, RNEasy Mini kit (Qiagen) and TRIzol LS (with and without filtering of the 10% feaces suspension at a 45 μm pore size)) were examined by NASBA. No amplification signal was observed for RNA from any of the extraction methods, indicating the RNA extraction method was not the main cause of amplification failure.

Furthermore, inhibition was studied using an internal amplification control provided with the NASBA kit. This control was added to the samples to examine whether the absence of detectable amplification products was due to inhibition. In several undiluted and 10-fold diluted RNA samples inhibition was observed. Further serial 10-fold dilutions of the RNA diminished inhibition, whereas amplification still was not detected despite the presence of HEV RNA as confirmed by conventional RT-PCR. Therefore, inhibition was not considered the main cause of amplification failure by NASBA.

5.1.1.4 Conclusions

Despite the analyses of a large number of primers and probe combinations, different NASBA conditions and alternative amplification and detection methods (PCR on NASBA products, NASBA on

RT-PCR products, use of internal amplification controls), it remains unclear why no NASBA amplification products of HEV RNA were detected. Therefore, it was decided to postpone further activities on the development of a real-time NASBA for the detection of HEV RNA, until new requirements arise for which the NASBA system is indispensable.

5.1.2 Quantitative real time RT-PCR

The shorter hands-on time for real-time RT-PCR as compared to conventional RT-PCR, however, advocates the development of real-time assays with a sensitivity equal to or greater than that of the currently employed assay. Several real-time RT-PCRs have been described for HEV (Mansuy et al., 2004; Orrù et al., 2004; Enouf et al., 2006; Jothikumar et al., 2006; Li et al., 2006b). Except for the assay described by Li et al. (2006b), we compared all other assays directly to the conventional RT-PCR to assess its applicability for HEV detection. The results are described below.

5.1.2.1 Materials and methods

Specifications of the protocols from the literature and comparison are presented in Table 7. Primer- and probe-sequences were as described by the authors. The synthetic positive control described by Orrù et al. (2004) was used for that assay. The reverse trancription procedure that was reported by Mansuy et al. (2004) was replaced with the reverse transcription procedure that was employed for the conventional HEV RT-PCR. The reverse primer of in the protocol by Enouf et al. (2006) was replaced by the reverse primer of the Jothikumar-protocol (Jothikumar et al., 2006). Furthermore, the protocol described by Enouf et al. (2006) is originally based on a separate reverse transcription, but the one-step protocol as described by Jothikumar et al. (2006) was used, because a one-step procedure is preferred given the shorter hands-on time required.

The most sensitive real-time RT-PCR protocol was further optimized by examining the effect of a reverse-primer concentration twice that of the forward-primer concentration (250 nM versus 500 nM and 300 mM versus 600 nM), altered probe concentration (100 nM versus 150 nM), altered volume of RNA examined (5 μl of RNA in stead of 2 μl) and altered forward primers. For the latter, two primers were developed that were located closer to the 5'-terminus of the genome, with sequences 5'-GGCCGGYCAGCCGTCTGG-3' (FHEVm) and 5'-TTCTGCCTATGCTGCCCGCGCCA-3' (FHEVm2), respectively.

5.1.2.2 Results

The protocols described by Orrù et al. (2004), Mansuy et al. (2004), and Enouf et al. (2006) were not as sensitive as the conventional RT-PCR and therefore unattractive to implement. In contrast, the protocol described by Jothikumar et al. (2006) was more sensitive than the conventional RT-PCR. Further optimization of that protocol yielded an increased sensitivity by analyzing 5 μl of RNA compared to the prescribed 2 μl (with the volume of the enzyme-mix being increased proportionally). The additionally examined primers (FHEVm and FHEVm2) did not increase the sensitivity of the assay, nor did altered primer concentrations. After optimization, 588 of the 591 faecal samples that were initially examined by the conventional RT-PCR were re-examined for HEV RNA with the real-time RT-PCR to estimate the increased sensitivity. Of the retested faecal samples, 278 samples tested negatively in both assays and 260 samples tested positively in both assays. Twelve of the conventional RT-PCR positive samples were negative by real-time RT-PCR; 38 of the real-time RT-PCR positive samples were negative by conventional RT-PCR. Interestingly, 29 of these 38 samples had a C_t >37, indicating a low HEV titer. The lowest C_t of samples that tested negative in the conventional RT-PCR was 35.6.

Table 7. Specifications of the real-time RT-PCRs evaluated for HEV detection and conclusion regarding its use.

Protocol	Principle of DNA detection	Apparatus	One-step reaction?	Conclusion
Orrù et al., 2004	SyBR green	LightCycler 2.0	Yes	An amplification signal in the non-template control was consistently observed. The melting curve analysis did not enable discrimination between positive samples and the non-template controls.
Mansuy et al., 2004	Probe hybridization	LightCycler 2.0	No	The sensitivity of the assay was lower compared to the conventional RT-PC.
Enouf et al., 2006	Probe hybridization	LightCycler 480	No	The sensitivity of the assay was lower compared to the conventional RT-PCR.
Jothikumar et al., 2006	Probe hybridization	LightCycler 480	Yes	More sensitive detection of HEV RNA compared to the conventional RT-PCR after slight modifications to the described protocol (described by Bouwknegt et al. (2009)).

5.1.2.3 Conclusions

HEV RNA detection according to the protocol described by Jothikumar et al. (2006), modified to examine 5 μl of RNA in stead of the reported 2 μl, was found to be more sensitive than the conventional RT-PCR used or any of the other examined real-time RT-PCR protocols (Table 7). Therefore, subsequent HEV detection and examination of environmental sources will be based in future experiments on the modified Jothikumar-protocol as described (Bouwknegt et al., 2009).

5.2 HEV detection in potential sources

5.2.1 Animals: game

At present pig, wild boar and deer are suspected sources of foodborne zoonotic transmission of HEV. The presence of HEV in Dutch pigs has been studied extensively (Bouwknegt et al., 2007; Rutjes et al., 2007). Nothing is known about the presence of HEV in the Netherlands in the other suspected sources. Because raw or undercooked wild boar or deer meat is more commonly eaten than raw or undercooked pork, the prevalence of HEV in these sources was studied to be able to estimate public health risks.

5.2.1.1 Material and methods

Sample collection

To be able to perform an extensive serological survey to study the prevalence of HEV in wildlife in the Netherlands, wild boar, red and roe deer were hunted on the Veluwe, a wilderness area consisting of woodland, heath land, lakes and dunes. The area contains a game reserve with many endangered plant and animal species. Wild boar (*Sus scrofa*) sera were collected from 2005 till 2008, in numbers varying from 25 in 2008 to 500 in 2005 (Table 8). Furthermore, in total 38 red deer (*Cervus elaphus*) sera and

eight roe deer (*Capreolus capreolus*) sera were collected between 2006 and 2008. Paired serum, faeces, liver and muscle (diaphragm) samples were collected from part of the studied wild boars and from all red and roe deer, although not in all cases all four different matrices were obtained.

Sample preparation

Blood

Blood samples were gathered from the abdominal cavity in a 50 ml tube. To collect serum from the samples, they were incubated at room temperature to clot and centrifuged. RNA was extracted from the sera by using the Qiamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted in a volume of 60 µl.

Faecal samples

Faecal samples were stored in 15 g/L of Trypton Soya broth (Oxoid CM 129) and 10% glycerol at -70 ºC until testing. For the extraction of viral RNA the samples were resuspended in Hanks balanced salt solution (Bibco BRL, Breda, the Netherlands) to a final concentration of 1%. The suspensions were centrifuged at 3000 *g* for 20 minutes. RNA was extracted using the Qiamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted in a volume of 60 µl.

Liver and muscle tissues

Three randomly selected pieces of approximately 1 cm^3 from the inner part of each liver were homogenized roughly with sterile surgical blades. Liver tissue (250 mg) was disrupted by the addition of 4 grams of Zirconia beads (1.0 mm; BioSpecProducts, Inc., Bartlesville, Okla.) and 1 ml of buffer L7 (Guanidine Thio Cyanate, 0.1M Tris-HCl at pH=6.4, 0.2 M EDTA, Triton X-100, 1 mg/ml Alpha-Casein (Sigma, Zwijndrecht, the Netherlands)) in a 2-ml Eppendorf tube using the Hybaid Ribolyser Cell Disrupter (Hybaid, Ashford, UK). The tissue was homogenized for 80 s at 4.0 m/s. The homogenate was left to settle, after which the lysate was collected from the zirconia beads. RNA was extracted according to Boom et al. (1990) using 100 µl of the remaining supernatant. Briefly, the lysate was added to 900 µl of L7, 10 µl silica beads (Sigma, Roosendaal, the Netherlands) and Zirconia beads, to be able to disrupt aggregates of silica beads if necessary. After incubation for 30 min at room temperature and centrifugation the silica beads were washed twice with L7 buffer, twice with 70% ethanol and once with acetone and dried at 56 ºC. The RNA was eluted from the silica beads by adding 35 µl elution buffer and incubation at 56 ºC for 30 minutes.

Detection of HEV-specific antibodies by ELISA

HEV-specific antibodies in sera from wild boar, red deer and roe deer were detected by the species independent double antigen sandwich (das) ELISA supplied by MP Biomedicals Asia Pacific Pte Ltd. in Singapore (MPB HEV dasELISA) (Hu et al., 2008). In this assay, 20 µl of heat-inactivated serum (30 minutes, 56 ºC) was analyzed in a five times dilution. Because of the dasELISA concept, HEV-reactive IgG as well as IgM and IgA is detected. For human samples, the assay was shown to have an overall specificity of 98.9%. The positive predictive value and negative predictive value were 98.1% and 100%, respectively (Hu et al., 2008). Previously, the assay has been shown to be successful in the detection of HEV-specific antibodies of both human and pig (*Sus scrofa domestica*) origin, confirming species independency (Hu et al., 2008).

Detection of HEV RNA by RT-PCR

Conventional RT-PCR

As a target for single-round amplification the conserved open reading frame (ORF) 2 region that codes for the viral structural proteins was used. Reverse transcription (RT)-PCR was performed with primers HEV ORF2-s1-3 (5'-GACAGAATTRATTTCGTCGGCYGG-3') and HEV ORF2-a1-3 (5'-CYTGCTCRTGYTGGTTRTCRTARTC-3', which have been optimized for detection of HEV genotype 3 species. Positions of the primers on strain SAR-55 (GenBank accession no. M80581) are 6270-6293 and 6442-6466, respectively. The specificity of the detected HEV signal was confirmed by hybridization of RT-PCR products as described previously (Van der Poel et al., 2001). An internal control (IC) RNA was included in the RT-step to monitor for inhibition of the RT-PCR reaction as previously described (Rutjes et al., 2007).

Real-time RT-PCR

Real-time detection of HEV RNA was performed as described previously (Bouwknegt et al., 2009).

Confirmation of detected RT-PCR products

HEV RT-PCR products positive by Southern blot hybridization or by real-time RT-PCR were cloned into a pCRII-TOPO vector (Invitrogen, Breda, the Netherlands) and analyzed for the presence of the correct insertion size by direct PCR amplification with M13 forward and M13 reverse primers supplied by the manufacturer. PCR products of the correct size were purified with the PCR purification kit and sequenced with the Big Dye Terminator Cycle Sequencing Ready Reaction. Nucleotide sequences were edited by Bionumerics (V4.0 Applied Maths, Kortrijk, Belgium). Multiple sequence alignment was done on either a 148 nucleotide fragment of ORF2 obtained by conventional RT-PCR or on a 34 nucleotide fragment of ORF3 obtained by real-time RT-PCR.

5.2.1.2 Results

Detection of HEV-specific antibodies in sera from wild boar, red deer and roe deer

As a result of the species independent concept of the dasELISA, sera from wild boar, red and roe deer, representing three different species: *Sus scrofa*, *Cervus elaphus* and *Capreolus capreolus*, respectively, were analyzed for the presence of HEV-specific antibodies by this species independent ELISA. As shown in Table 8, in 2005 and 2007 up to 500 wild boar sera have been analyzed and resulted in a seroprevalence of 28 ± 1% and 38 ± 2%, respectively. A mean seroprevalence of 33% (335/1029) was observed within the period 2005 – 2008. Red and roe deer were analyzed in smaller numbers, resulting in a seroprevalence of 11% (4/38) for red deer. HEV-specific antibodies were detected once in roe deer, but the sample size was too small to be conclusive.

Table 8. HEV das-ELISA results obtained in sera from wild boar, red deer and roe deer between 2005 – 2008.

	2005		2006		2007		2008	
	N	Pos (%)	N	Pos (%)	N	Pos (%)	N	Pos (%)
Wild boar	499	142 ± 6 (28 ± 1%)	37	11± 2 (30 ± 5%)	469	177 ± 11 (38 ± 2%)	24	6 ± 1 (25 ± 3%)
Red deer	nd[1]	nd	8	0 (0%)	20	4 ± 1 (18 ± 4%)	10	2 ± 1 (15 ± 7%)
Roe deer	nd	nd	4	1 ± 0.7 (13 ± 18%)	nd	nd	4	0 (0%)

[1] nd: not determined

Table 9. HEV RT-PCR results obtained in samples from wild boar, deer and roe between 2005 – 2008.

Matrix	Wild boar		Red deer		Roe deer	
	conv	real-time	conv	real-time	conv	real-time
Serum	2/105 (2%)	5/105 (5%)	0/38 (0%)	2/38 (5%)	0/8 (0%)	0/8 (0%)
Faeces	1/93 (1%)	2/93 (2%)	0/35 (0%)	1/35 (3%)	0/8 (0%)	0/8 (0%)
Liver	1/77 (1%)	2/102 (2%)	0/29 (0%)	1/39 (3%)	0/4 (0%)	0/8 (0%)
Muscle	0/39 (0%)	0/64 (0%)	0/28 (0%)	2/39 (5%)	0/2 (0%)	0/6 (0%)
Tot pos animals	3/106 (3%)	8/106 (8%)	0/38 (0%)	6/39 (15%)	0/8 (0%)	0/8 (0%)

Detection of HEV RNA wild boar, red deer and roe deer

The presence of HEV RNA in serum, faeces, liver and/or muscle was studied in the collected animals by both conventional and real-time RT-PCR. Conventional RT-PCR was performed because of the availability of an internal control RNA to control for inhibition of the PCR, which is an important issue in molecular analyses of environmental samples (Rutjes et al., 2007). Furthermore, the PCR product is larger and thus more suitable for phylogenetic analysis than the sequence obtained by real-time PCR, 148 nt versus 34 nt, respectively. On the other hand, real-time RT-PCR appeared to be more sensitive than conventional RT-PCR, as is shown by the results presented in Table 9. By conventional RT-PCR, a HEV prevalence of 3% was observed for wild boar, and no HEV RNA was detected in red and roe deer. By real-time RT-PCR, the prevalence in wild boar increased to 8%. HEV RNA was detected most often in sera, but also faeces and liver of wild boar were shown to contain detectable levels of HEV RNA. By real-time RT-PCR HEV RNA was detected in 15% of the red deer samples, serum and muscle samples were most frequently positive. In none of the roe deer samples HEV RNA was detected. From these results it was concluded that hepatitis E virus circulates in both the wild boar and the red deer population inhabiting the Veluwe.

Of the samples in which HEV RNA was detected by conventional RT-PCR, sequences were obtained from one liver, one faeces and one serum sample (Table 10) by cloning of the PCR product. HEV sequences were obtained from five clones derived from wild boar liver 05-54, from 11 clones of serum and 7 clones of faeces derived from wild boar 05-21. No HEV sequences were obtained from the serum of the third HEV positive wild boar 05-20. Phylogenetic analysis of the obtained sequences revealed that the detected HEV sequences clustered with genotype 3c sequences that were isolated from Dutch pig farms in 2005. Interestingly, 16 of the 18 clones of wild boar 05-21 were 100% identical with 3 of the 5 clones of wild boar 05-54 in a fragment of 148 nucleotides of ORF2 (Figure 3).

Table 10. Ct values of samples in which HEV RNA was detected by real-time RT-PCR.

	Wild boar				Red deer		
	Ct value				Ct value		
Sample	Undiluted RNA	10^{-1} diluted RNA	Tissue	Sample	Undiluted RNA	10^{-1} diluted RNA	Tissue
05-11	38.15	Neg	Serum	07-01	Neg	40.00	Serum
05-20*	34.90	35.01	Serum	07-09 #	38.30	Neg	Serum
05-21*	30.14	30.63	Serum	07-11	40.00	Neg	Faeces
05-21	32.72	32.37	Faeces	07-12 #	Neg	40.00	Muscle
05-54* #	40.00	37.01	Liver	07-17	Neg	40.00	Muscle
06-113	Neg	36.67	Serum	08-28	38.56	Neg	Liver
07-15	Neg	40.00	Serum				
08-12	38.25	Neg	Liver				
08-21	Neg	39.49	Faeces				

* Positive by conventional RT-PCR; # Positive by dasELISA
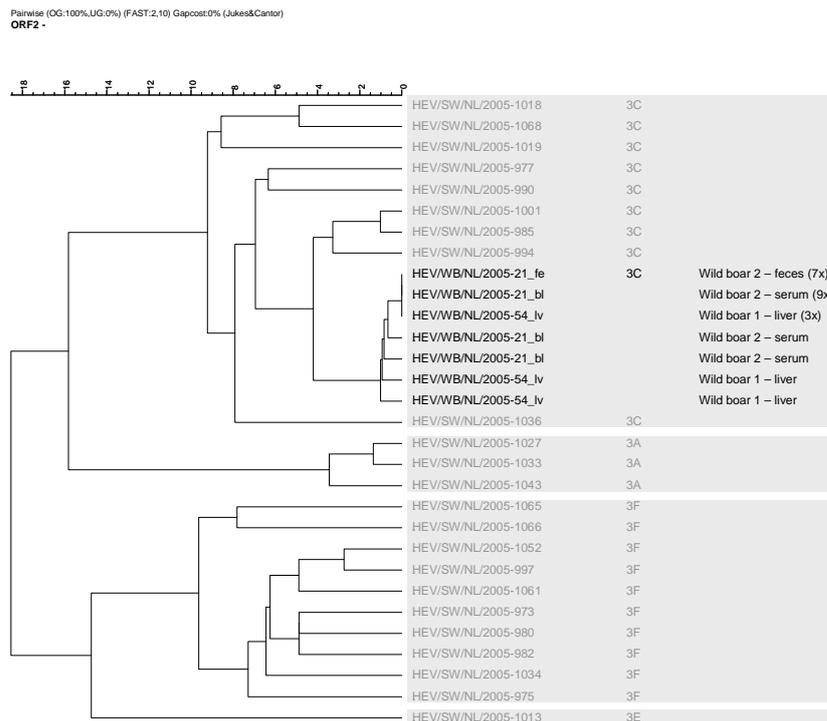
Figure 3: Phylogenetic relationships between Dutch pig and wild boar strains of hepatitis E virus, based on a 148 nt sequence of ORF2 (nucleotides 6270 to 6464 of the M80581). Newly identified wild boar sequences (black) are depicted and are compared with Dutch sequences of swine isolated in 2005 (grey) representing the different genotype 3 clusters that have been detected in the Netherlands, 3a, 3c, 3e and 3f. Also depicted is the numbers of identical clones of which HEV sequences were obtained.

5.2.1.3 Discussion and conclusions

The current study demonstrates by serological and molecular studies that HEV is present in wild boar and red deer on the Veluwe, suggesting that HEV is endemic in this area. The sequences isolated from wild boar clustered together with sequences obtained from Dutch pig farms in cluster 3c, a cluster which thus far is unique to the Netherlands (Rutjes et al., 2009b). The observed HEV prevalence in wild boar is in the range of prevalences observed elsewhere in Europe. In 5.3% of German wild boar sera HEV RNA was detected and in 19.6% and 25% of Spanish wild boar sera and Italian wild boar bile, respectively.

Although extraction methods for the isolation of HEV RNA from the different tissues have improved the removal of inhibitors substantially, such as optimization of the amount of input material and the addition of zirconia beads to disrupt aggregates of silica in liver and muscle samples, still a considerable part of the analyzed samples are inhibited, either in the undiluted or ten-fold diluted samples. The samples in which HEV RNA was detected by conventional RT-PCR were also positive by real-time PCR (Table 10). Obtained Ct values were generally lower in these samples than in samples positive only by real-time PCR. Although the real-time RT-PCR is thus more sensitive than the conventional PCR, the combination of RNA extraction and real-time PCR may still be improved. In an optimal real-time RT-PCR, Ct values raise a factor 3.3 by every ten-fold dilution of the RNA. The fact that Ct values are higher (or negative) in undiluted samples than in ten-fold dilutions of these samples indicates that inhibition of the amplification reaction occurred. Thus, not only the presence of an internal control RNA in a conventional RT-PCR indicates whether PCR inhibitors are present, also the behavior of Ct values in serial ten-fold dilutions is indicative for the presence of inhibitors.

The studied red deer were hunted in the region where HEV-positive wild boars were caught, suggesting that interspecies transmission may have occurred between red deer and wild boar. This was previously shown for wild deer and boar that were hunted in a shared habitat in Japan by high nucleotide similarities of 99.7% within a fragment of 7.2 kb of HEV RNA isolated from these animals (Takahashi et al., 2004). In roe deer, no HEV RNA was detected, which may be explained by the low number of studied samples.

This is the first report showing the presence of HEV in red deer samples in Europe. Although the number of analyzed samples is low, the data at least suggest a similar prevalence of HEV in wild boar and deer, suggest that consumption of raw or undercooked wild boar and red deer products may cause transmission of HEV and thus a public health threat.

5.2.2 Food: shellfish

Shellfish are filter feeders and, as a consequence of their feeding process, might concentrate human pathogens present in their growing waters in case of faecal contamination. European legislation prescribes the classification of shellfish harvesting areas into one of three categories (A, B or C) according to the degree of *Escherichia coli* present in whole shellfish flesh (Councel Directive 91/492/EEC) (Anonymous, 1991). However, compliance to category A (less than 230 *Escherichia coli* in 100 g of whole shellfish flesh) does not prevent events of enteric disease outbreaks associated with the consumption of shellfish (Potasman et al., 2002; De Roda Husman et al., 2007). This suggests that the faecal indicator bacteria *Escherichia coli* is not suitable to predict the presence of pathogenic viruses. Because it was demonstrated that HEV is present in Dutch surface waters, the virus may accumulate in shellfish and may thus cause a potential health risk, especially when eaten raw. To be able to estimate this health risk, the presence of the pathogenic virus HEV was studied in shellfish derived from Dutch category A growing areas.

5.2.2.1   Material and methods

Samples

Japanese oysters (*Crassostrea gigas*) (n=104) and mussels (*Mytilus edulis*) (n=104) were collected monthly or biweekly in commercial harvesting areas in the Netherlands from June 2007 till December 2008. Digestive glands derived from six oysters or six mussels were pooled and 250 mg of the pooled samples were analyzed for the presence of HEV RNA. Alternatively, digestive glands were studied individually.

Extraction of viral RNA and RT-PCR detection

Seeded digestive glands (2.0 g) were chopped with a razor blade and transferred into a centrifuge tube. An equal volume of Proteinase K solution (30 units per mg) was added followed by incubation at 37 ºC for 60 min. A second Proteinase K incubation was carried out at 65 ºC for 15 min prior to centrifugation at $3000 \times g$ for 5 min as described previously (Jothikumar et al., 2005). RNA was extracted using NucliSens magnetic silica particles (bioMérieux, Boxtel, the Netherlands) according to the instructions of the manufacturer, using 500 µl of the remaining supernatant, approximately corresponding to 400 mg – 600 mg of digestive gland tissue. Nucleic acids were recovered from the particles during a 5 minute incubation period at 60 °C, using 50 µl elution buffer (Rutjes et al., 2005). Real-time detection of HEV RNA was performed as described previously (Bouwknegt et al., 2009).

5.2.2.2   Results

Analysis of commercial oysters and mussels

RNA was extracted from a pool of six oyster digestive glands and a pool of six mussel digestive glands as well as from individually isolated digestive glands from oysters and mussels. HEV RNA was detected by real-time RT-PCR. As is shown in Table 11, in pooled digestive glands of mussels, once HEV RNA was detected (June 2007), in pooled digestive glands of oysters, no HEV RNA was detected. The HEV RNA positive digestive glands of individually analyzed oysters and mussels were detected on three other sampling dates: September 2007 (1 mussel), October 2007 (one oyster, one mussel) and December 2008 (two oysters, one mussel). These data have been obtained by real-time PCR analyses and still has to be confirmed by sequencing analyses of the PCR products.

5.2.2.3   Discussion and conclusion

HEV RNA was detected in oysters and mussels from four different sampling dates. On three of these sampling dates, HEV RNA was detected in individually analyzed digestive glands only, not in the pooled samples. On two of these sampling dates, in more than one sample HEV RNA was detected, suggesting that shellfish from those sampling dates was more contaminated than from the dates that no HEV RNA was detected, which may be reflected in the microbial water quality. On one sampling date a pool of digestive glands from mussels was found to be positive for HEV RNA. Unfortunately, no individual shellfish was analyzed on this date. Our data however, suggest that the sensitivity of detection increased when digestive glands from oysters and mussels were analyzed individually. In literature, there is a tendency to analyze pooled samples, because the pooled sample will be more representative than individual shellfish. Also in the international standard on detection of viruses in foods, which is currently developed by a working group of the European Committee for Standardisation, it is prescribed that 2 grams of a pool of at least 10 digestive glands should be analyzed.

Table 11. Presence of HEV RNA in pooled and individually analyzed digestive glands of oysters and mussels that were harvested between June 2007 and December 2008.

	Oysters				Mussels			
	Pooled		Individual		Pooled		Individual	
	N	positive	N	Positive*	N	Positive*	N	Positive*
2007	9	0/9	21	1/21	9	1/9	21	2/21
2008	11	0/11	63	2/63	11	0/11	63	1/63

* PCR products needs to be confirmed by sequencing analyses of the PCR products.

Oysters and mussels from all sampling dates have been analyzed for the presence of the indicator bacteria *E. coli* in 100 g of shellfish flesh. In all cases, the levels of *E. coli* complied with the norm used for category A harvesting areas, indicating that less than 230 *E. coli* colony forming units were present in 100 g of whole shellfish flesh. No correlation was observed between the presence of *E. coli* bacteria and the presence of HEV RNA in shellfish. This confirms that the faecal indicator bacteria *E. coli* is not suitable to predict the presence of pathogenic viruses. Instead, testing for the presence of either the pathogenic virus itself or an indicator virus, such as F-specific RNA bacteriophages (Havelaar et al., 1993), human Adenoviruses (hAdV) (Pina et al., 1998; Formiga-Cruz et al., 2003) and human Circoviruses (huCV) (Myrmel et al., 2004; Haramoto et al., 2005), may be considered.

5.2.3   Drinking water

Partially treated surface waters for the production of drinking water, such as water in storage reservoirs that has been treated by infiltration in the dunes, may become contaminated by the re-introduction of faecal matters from animals. In the current study, two storage reservoirs were studied. Reservoir A is located in the dunes and may be contaminated with HEV, because of excretion of the virus by wildlife. Red and roe deer walk freely in the dunes, whereas cattle and sheep are kept at a distance of at least 100 m from storage reservoir A, including supply and production canals. Storage reservoir B is fed with groundwater and is surrounded by two ponds which may contaminate storage reservoir B. No red and roe deer are present in the area of reservoir B, but small animals like rodents and birds are likely to be present. Because the presence of HEV in partially treated drinking water may be a threat to public health, both reservoirs were studied for the presence of HEV RNA.

5.2.3.1   Materials and methods

Sampling

Water samples of 20 liters were taken from two storage reservoirs for drinking water production, A and B. Samples were taken in February 2008 and were concentrated by filtration through a cross-flow ultrafilter (Hemoflow HF80S; Fresenius Medical Care, Bad Homburg, Duitsland) until the volume was reduced to 250 ml. Possible attached viruses were eluted from the filter with an elution buffer containing 0.1% glycine. A volume of 10% beef extract (Difco Laboratories, Detroit, USA) was added to the eluate until a final concentration of 3% beef extract was obtained. The eluate was further concentrated by ultrafiltration (UF) (Rutjes et al., 2005). For ultrafiltration, a cellulose-acetate filter (NMWL 10,000) was used under high pressure (3 bar). This final UF-concentrate was stored at –70 °C until subjected to RNA extraction.

RNA extraction and RT-PCR

The ultrafiltered concentrate of the water samples was subjected to a NucliSens-based RNA isolation (Rutjes et al., 2005). After the addition of 1 ml and 5 ml of eluate and 0.2 ml or 1 ml of ultrafiltered concentrate to 1 ml, 5 ml, 1 ml and 9 ml, respectively, of NucliSens lysis buffer (bioMérieux, Boxtel,

the Netherlands) and 50 µl of NucliSens magnetic silica particle suspension (bioMérieux, Boxtel, the Netherlands), RNA was isolated as described by the manufacturer. The NucliSens miniMAG instrument (bioMérieux, Boxtel, the Netherlands) was used to collect and wash the magnetic silica particles. Nucleic acids were recovered from the particles during a 5 minute incubation period at 60 °C, using 50 µl elution buffer (Rutjes et al., 2005). Real-time detection of HEV RNA was performed as described previously (Bouwknegt et al., 2009).

5.2.3.2 Results

In both storage reservoirs HEV RNA was detected by real-time RT-PCR. Ct-values varied from 37.07 in storage reservoir A in the analysis of 1 ml of ultrafiltered concentrate to >40 in the analysis of 1 ml and 5 ml of eluate in storage reservoir B. In this reservoir no HEV RNA was detected in the analyzed UF-concentrate. HEV RT-PCR signals still have to be confirmed by sequencing analyses.

5.2.3.3 Discussion and conclusion

Assuming that the observed RT-PCR signals can be confirmed by sequencing analysis as being hepatitis E virus, more HEV RNA was detected in storage reservoir A, than in storage reservoir B. This may be explained by the fact that storage reservoir A is more faecally polluted than reservoir B because of the presence of red and roe deer, indicating that more virus may be present. An alternative explanation may be found in the fact that the volume reduction by concentration of water from storage reservoir A was less efficient (330×) than volume reduction from reservoir B (1000×). Due to the less efficient concentration, less PCR inhibitors may have been isolated, which may have a positive effect on the sensitivity of the RT-PCR. To test this hypothesis, more diluted RNA should be analyzed by RT-PCR to see whether dilution of the sample and thus dilution of the possible inhibitors positively influence the efficiency of the RT-PCR. More samples taken throughout the year should be analyzed to get more detailed information on the presence of HEV. The fact that HEV RNA was detected in partially treated drinking water indicates that a public health risk may exist when further treatment is not sufficient to remove or inactivate the viruses to an acceptable level as can be estimated by quantitative microbial risk assessment (QMRA).

5.2.4 Bathing water

Exposure to microbiologically contaminated surface water may have adverse health effects and may result in gastroenteritis, fever, skin, ear and eye complaints or more severe illnesses, such as hepatitis and meningitis (WHO, 2003). In the Netherlands, the presence of pathogenic viruses like norovirus, rotavirus and enterovirus has been demonstrated in several surface waters, such as river water (Lodder and De Roda Husman, 2005; Rutjes et al., 2005; Rutjes et al., 2006; Rutjes et al., 2009a) and urban canals (Schets et al., 2008). Because the presence of HEV RNA in surface water has not been described to date, the presence of HEV RNA was studied in the river Meuse. Detection of HEV RNA in surface waters will indicate that transmission by recreation related exposure should be taken into consideration.

5.2.4.1 Materials and methods

Sampling

Water samples of 600 liter were taken from the river Meuse in Eijsden at the border with Belgium in the southern part of the Netherlands. Samples were taken monthly from September 2004 to July 2005. Samples were concentrated by a conventional filter adsorption-elution method by the addition of magnesium chloride and reducing the pH to 3.8 (Lodder and De Roda Husman, 2005). Viruses were eluted from the filter with an elution buffer (pH 9.0) containing 3% beef extract (Difco Laboratories, Detroit, USA) and 0.05 M Tris (Bisolve, Valkenswaard, the Netherlands). The typical retentate volume

was approximately 1,800 ml and was neutralized with a concentrated acetic acid buffer (pH=5.0) to a final pH of approximately 7.4. The eluate was further concentrated by ultrafiltration or two-phase separation (Lodder and De Roda Husman, 2005). For ultrafiltration, a cellulose-acetate filter (NMWL 10,000) was used under high pressure (3 bar). The ultrafilter was rinsed with 3% beef extract (pH=9.0). This final UF-concentrate was stored at −70 °C until subjection to RNA extraction. For two-phase separation, 1% (w/v) Dextran T40, 10% (w/v) PEG 6000, 0.2 M NaCl, and 10 mM phosphate buffer (pH=7.2) were added to 650 mL eluate, and mixed for 1 h at 4 °C on a horizontal shaker. The suspension was then transferred to a separation funnel and left overnight at 4 °C. After separation, the bottom phase and the interphase were harvested. Further purification was done by spin-column gel chromatography using Sephadex G200 (ICN, Zoetermeer, the Netherlands), and by ultrafiltration in a Centricon 100 micro concentrator, 100 000 MW cut-off (Amicon, Dronten, the Netherlands). The average retentate volumes were 1-5 mL.

RNA extraction

The ultrafiltered concentrate of the water samples was subjected to a NucliSens-based RNA isolation (Rutjes et al., 2005). After the addition of 12.5 µl of ultrafiltered concentrate to 500 µl of NucliSens lysis buffer (bioMérieux, Boxtel, the Netherlands) and 50 µl of NucliSens magnetic silica particle suspension (bioMérieux, Boxtel, the Netherlands), RNA was isolated as described by the manufacturer. The NucliSens miniMAG instrument (bioMérieux, Boxtel, the Netherlands) was used to collect and wash the magnetic silica particles. Nucleic acids were recovered from the particles during a 5 minute incubation period at 60 °C, using 50 µl elution buffer (Rutjes et al., 2005).

Viral RNA was extracted from the retentate concentrated by two-phase separation by binding to size-fractionated silica beads (Sigma, Roosendaal, the Netherlands) in the presence of guanidinium isothiocyanate (GuSCN). Bound RNA was washed and eluted in 60 µl elution buffer as described (Lodder and De Roda Husman, 2005).

Molecular detection and typing of HEV RNA

Detection of HEV RNA was performed with RT-PCR and southern blot hybridization as described previously (Rutjes et al., 2007). Positive samples were typed by sequencing analysis. HEV RT-PCR products of the expected size were excised from a 2% agarose gel, purified with a Qiaquick gel extraction kit (Qiagen, Hilden, Germany) and sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Nucleotide sequences were edited and aligned by Bionumerics (V4.0 Applied Maths, Kortrijk, Belgium) using the neighbour joining method after multiple sequence alignment.

5.2.4.2 Results

Two out of 12 water samples from the Meuse were positive for the presence of HEV RNA. The first HEV positive sample was detected in September 2004, in RNA extracted from ultrafiltered concentrate, the second positive sample was detected in March 2005 in RNA isolated from the retentate obtained by two-phase separation. HEV concentrations detected in September 2004 and March 2005 were estimated to be 99.0 (4.6 – 930.9) PDU/L and 1.9 (0.090 – 18.2) PDU/L, respectively. Sequencing analysis showed that the two detected HEV sequences clustered with genotype 3 sequences. The sequence originating from the sample taken in 2004 clustered closely together (<6% divergence) with subgroup 3f, the sequence from 2005 with subgroup 3e (<4% divergence).

5.2.4.3 Discussion and conclusion

In the Netherlands per year approximately 20 cases of hepatitis E are reported, which may be an underestimation. Often the source of infection is unknown and to date it is unclear if surface waters

play a role in transmitting these viruses to humans in the Netherlands. The estimated HEV RNA concentrations were 99.0 (4.6 – 930.9) PDU/L and 1.9 (0.090 – 18.2) PDU/L in September 2004 and March 2005, respectively. The difference in concentration is likely to be the result of the RNA extraction procedure used. In the past, RNA was extracted from concentrate that was obtained by a method based on a two-phase separation using dextran T40 and PEG 6000 and silica beads. In 2005, the method for RNA extraction from concentrated water samples was optimized by using UF-concentrate as the starting material for extraction. The silica used for RNA extraction was replaced by magnetic silica beads (Rutjes et al., 2005). Using this optimized method, estimated RNA concentrations detected in the samples increased at least 100 to 500 times.

The presence of HEV RNA in a sample does not prove the presence of infectious viruses, but in the absence of a cell culture system, information on the presence of viral genomes obtained by detection by molecular methods is the best alternative to assess potential health risks. We recently demonstrated that in surface waters in 55% of the samples, rotavirus RNA was 1000 to 10000 times ($3 \log_{10} - 4 \log_{10}$) more abundantly present than infectious rotavirus particles. In the remaining 45% of the samples, rotavirus genomes were less than 1000 times ($< 3 \log_{10}$) more abundantly present, suggesting that these samples were more recently contaminated than the previous samples (Rutjes et al., 2009a). This relation between infectious virus and virus genomes was found to be similar for enterovirus in different source waters (Rutjes et al., 2005). Assuming a similar relation between infectious HEV and HEV RNA ($3 \log_{10}$ difference), infectious virus concentrations in surface water may be around 0.1 infectious virus particle per liter. Thus, the presence of HEV RNA in surface waters may indicate a possible risk when these waters are used for shellfish culture, drinking water production or recreational purposes.

6   Hazard characterization

Hazard characterization involves modeling of the infection risk given a certain dose. This so-called dose-response relation can be derived from human volunteer experiments, in which humans are given known doses of a pathogen. Given the ethical constraints in doing these experiments, no such data are available for HEV.

Another approach for constructing a dose-response relation is by obtaining quantitative data from case patients from outbreaks or sporadic infections. The ingested dose, however, is generally difficult to quantify, given the uncontrolled conditions during which the infection was acquired. Furthermore, low-dose cases, which are extremely important in modeling the dose-response relation, are difficult to observe during outbreaks, because low-doses likely have a higher average probability of causing subclinical infections.

A third approach is the use of an animal model. An exponential dose-response relationship has been described for pigs following intravenous inoculation (Bouwknegt et al., in preparation). This model estimated that the probability of infection per HEV particles (r) was 1.3×10^{-2}. This model was adjusted to represent the probability of infection following oral inoculation by reducing the probability of infection per HEV particle by a factor of 10^4. This factor was based on the observation that faecal-orally transmitted HEV is at least 10,000 times less infective than iv inoculated (Kasorndorkbua et al., 2002). The probability of infection (P_{inf}) per infectious HEV particle given a certain ingested HEV dose (D) is modeled by:

$$P_{inf} = 1 - e^{-r \cdot D}$$

Whether the infectivity r of HEV for humans and pigs are comparable is unknown. This may not be the case, considering the rapid spread of HEV that is observed among pigs and not among humans. In contrast, this difference may be related to the sanitary conditions in which pigs and humans exist. To account for differences in r between pigs and humans, scenarios can be evaluated in which r is for instance reduced 10^2-fold and 10^6-fold.

7   Discussion

The work described in this report is the initial phase of the quantitative microbiological risk assessment for HEV in the Netherlands. Hepatitis E virus infections are acquired by humans in the Netherlands and lead to clinical cases of hepatitis E (Widdowson et al., 2003; Herremans et al., 2007; Borgen et al., 2008). The sources for these cases are not identified. Furthermore, these identified cases are those that report to hospitals, and the total number of HEV infected persons is expected to be higher. The potential HEV sources for human HEV infection can be identified by screening of environmental samples. By subsequently estimating–per identified source–the levels of HEV to which humans are exposed, potential risk factors can be identified. This procedure of quantitative microbiological risk assessment (QMRA) (ILSI Risk Science Institute Pathogen Risk Assessment Working Group, 1996) omits the necessity of (scarcely identified) HEV cases, as is required for epidemiological studies. In addition, those routes that contribute most to human exposure to HEV as identified by QMRA can be targeted with effective intervention measures.

The described risk profile and data collection indicate that HEV is present in many environmental and animal sources, both in the Netherlands and worldwide. The potential sources for the Netherlands include domestic pigs, wild boar, wild deer, surface water, bathing water, oysters and mussels (and potentially other bivalves). Hence, given this widespread presence of HEV, many potential sources for exposure of humans to HEV exist. The associated routes of transmission that lead to human exposure to HEV may include direct contact with HEV infected animals, consumption of pork or game products, consumptions of fruits and vegetables that may be contaminated by HEV from irrigation with surface water, consumption of unboiled drinking water, and swimming in surface or bathing water.

The data fed into the risk assessment need to be quantitative data. When presence of HEV is determined by conventional RT-PCR, methods are available to obtain the most probable number and associated 95% interval (Westrell et al., 2006). When presence of HEV is determined by real-time RT-PCR, the concentration is generally inferred from a standard curve that is based on reference samples with a presumed known target-concentration. This procedure yields a point estimate of the target concentration, but is subject to bias (Bustin, 2002). For instance, the concentration of target in the reference samples is impossible to quantify. Therefore it is important to include sources of variation and uncertainty in the estimates of concentrations. A quantification control will be developed for real-time detection of HEV using the Jothikumar-protocol (Jothikumar et al., 2006). A statistical tool is subsequently required to adequately address sources of variation, such as pipetting variation, non-homogenous distribution of particles and inhibition during the RT-PCR reaction, to estimate the HEV concentration and the associated uncertainty.

To assess the exposure for each of the potential routes, further understanding of factors contributing to exposure to HEV within these routes is required. For instance, direct contact with HEV infected animal most likely leads to exposure to HEV for swine farmers, veterinarians, butchers, slaughterhouse personnel and hunters. In addition, the age of the contacted animals (related to the stage of infection for domestic pigs (Bouwknegt et al., 2009)) and the intensity of contact differs among people within these categories, leading to different exposure levels to HEV. Therefore, conceptual models that describe in more detail each of the exposure routes are required. Given the wide spectrum of potential transmission routes and the efforts required to conduct a proper risk assessment, the authors suggest to analyze one route associated with food (wild boar and deer meat), one route associated with water (drinking water

that is produced from surface water) and one route associated with direct contact with animals (domestic pigs). For these routes, conceptual models will be developed as the next phase of the QMRA. These models will identify the parameters that need to be quantified from data that may be obtained from literature or will be actively collected. Once completed, the exposure through each of these routes will be quantified, yielding insight in their importance for human HEV exposure. The approach could be followed for additional routes of exposure, and/or intervention measures can be developed to target effectively the reduction of exposure of humans to environmental HEV.

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