Hepatitis E virus infections in pigs

Transmission dynamics and human exposure

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Hepatitis E virus (HEV) of genotype 3 can be contracted in developed countries from currently unidentified sources. Zoonotic transmission of HEV from pigs has been suggested. The research described in the current thesis focused on the transmission dynamics of HEV among pigs and possible exposure of humans to porcine HEV. First, it was studied whether HEV can spread among pigs, a prerequisite to identify pigs as potential animal reservoir. HEV was shown to spread among pigs with an estimated R_0 of 8.8 (95% confidence interval: 4 – 19). To identify the transmission sources for porcine HEV, the course of infection in pigs was studied. This course was characterized by faecal HEV RNA excretion that started at day 7 (day 5 – 10) postexposure and lasted 23 (19 – 28) days, by viremia that started after 13 (8 – 17) days of faecal HEV RNA excretion and lasted 11 (8 – 13) days and by antibody development that was detected after 13 (10 – 16) days of faecal HEV RNA excretion. HEV RNA was also detected in several organs, including liver, and most interestingly in muscle samples that were proxies for pork at retail and in urine of contact-infected pigs. The latter finding suggests another possible transmission route for HEV. The assumed leading role of faeces in the transmission of HEV among pigs was therefore examined combining a quantitative exposure assessment and dose response modeling. This study showed that faeces is very likely to contribute to HEV transmission, although depending on the parameter values at most 94% of new cases may be caused by transmission sources other than faeces. Human exposure to porcine HEV in The Netherlands was examined for two routes: consumption of porcine livers and direct contact with pigs. HEV RNA was observed in 4 of 62 porcine livers, potentially leading to 1,800 (486 – 4,200) HEV-contaminated porcine livers to be consumed annually. The study focusing on direct contact with pigs showed that swine veterinarians had a higher estimated seroprevalence of anti-HEV antibodies compared to individuals from the general population (~11 versus ~2%, respectively). Non-swine veterinarians had an estimated seroprevalence of ~6%, but this estimate was not believed to be significantly different from the other two estimates. These data suggest that direct contact with pigs or being on the premises of pig farms is a risk factor for HEV infection. In conclusions, HEV was shown to spread among pigs, likely through faeces but possibly additionally through other matrices such as urine. Furthermore, it was shown that humans in The Netherlands can be exposed to porcine HEV. Evidence for human HEV infections being caused by porcine HEV, however, remains absent. Because HEV is also present in other Dutch reservoirs, such as wild boar and deer, and environmental sources, such as surface water, oysters and mussels, a comparative exposure assessment can guide further research and aid the assessment of the importance of domestic pigs in HEV exposure of humans in The Netherlands.

Table of Contents

Chapter 1

General introduction

1.1   Hepatitis E virus

Hepatitis E virus was characterized for the first time in 1990 [125] and has since been a public health concern to both developing and developed countries [123]. Until 1980, two forms of viral hepatitis were known: hepatitis A and hepatitis B. Hepatitis A was considered the epidemic variant of hepatitis caused by faecal-oral transmission of hepatitis A virus, whereas hepatitis B was considered the non-epidemic variant caused by transmission of hepatitis B virus through blood or sexual contact (parenteral transmission). However, other forms of viral hepatitis were suggested to exist, because serological assays excluded hepatitis A and hepatitis B for cases of viral hepatitis [120, 45]. These unidentified cases were referred to as non-A, non-B (NANB) hepatitis and were partly caused by parenteral transmission of an agent later defined as hepatitis C virus. For another part, these NANB-cases were caused by faecal-oral transmission of an infectious agent, which was shown later to be hepatitis E virus [149].

Hepatitis E virus (HEV) is a non-enveloped, positive-sense, single-stranded RNA virus of approximately 7.2 kilobases and the genome contains three open reading frames (ORFs) (Fig. 1) [122]. The polyprotein that is encoded by ORF1 comprises between 1691 and 1708 amino acids, depending on the isolate [136]. The partial proteins of ORF1 are coded from sequences that are homologous to those that code for *a.o.* methyltransferase and RNA-dependent RNA polymerase, important proteins for successful replication of the virus [122]. ORF2 encodes for the protein of the viral protein shell (capsid), which comprises 659 or 660 amino acids, depending on the isolate [136]. The ORF3 protein is the least conservative and comprises 122 or 123 amino acids, depending on the isolate [136]. The function of the ORF3 protein needs additional investigation, but the protein is suggested to be involved in intracellular immunosuppression [136, 157].

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* [37]. Hepatitis E viruses are classified into four genotypes, consecutively named 1 through 4
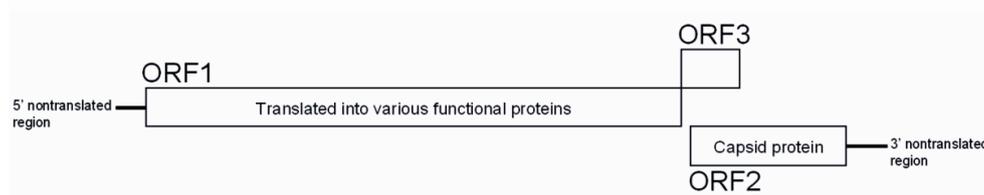

Figure 1. Genome organization of hepatitis E virus [based on ref. 177]

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[114]. 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 [55]. 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) [91]. 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.

The different HEV genotypes show a distinct geographical distribution [91]. 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.

1.2 Hepatitis E virus infection in humans

Clinical symptoms of hepatitis E virus infections 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 enlargement [122]. 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 [68, 122], although several chronic cases have been reported [49, 75]. Mortality rates among patients are generally <0.5%, but may reach up to 25% in pregnant women for at least genotype 1 [83]. The reasons for the high mortality rate in pregnant women are still unknown.

Since its discovery, hepatitis E virus is associated with large outbreaks of hepatitis E in developing countries. Predominantly inhabitants from Asian and African countries are infected by the virus due to poor sanitary conditions [122]. Especially during heavy rainfall, sewage overflow may contaminate surface water that is used as drinking water or as source

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[1] The definition of developed and developing countries used in this thesis is adopted from The World Factbook 2006 of the Central Intelligence Agency (CIA), available at www.cia.gov. Accessed March 26, 2008.

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to produce house water. As water is widely distributed and used, the number of people at risk is generally large, explaining the large-scale outbreaks of HEV in developing countries [163].

Despite the observed outbreaks only in developing countries, anti-HEV antibodies have been observed globally, including the developed countries (Fig. 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 [182, 183]. In 1998, the first HEV-sequence from a locally-acquired hepatitis E patient was obtained in the USA [84], 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 United Kingdom and Germany [60, 138, 184, 117, 145, 171, 6, 119]. Hence, HEV infections are also acquired locally in developed countries.

In The Netherlands, the first reports on hepatitis E cases date from 1992 with patients who contracted HEV presumably in Bangladesh and Pakistan [160, 162]. A subsequent serosurvey among blood donors in The Netherlands showed an estimated seroprevalence of
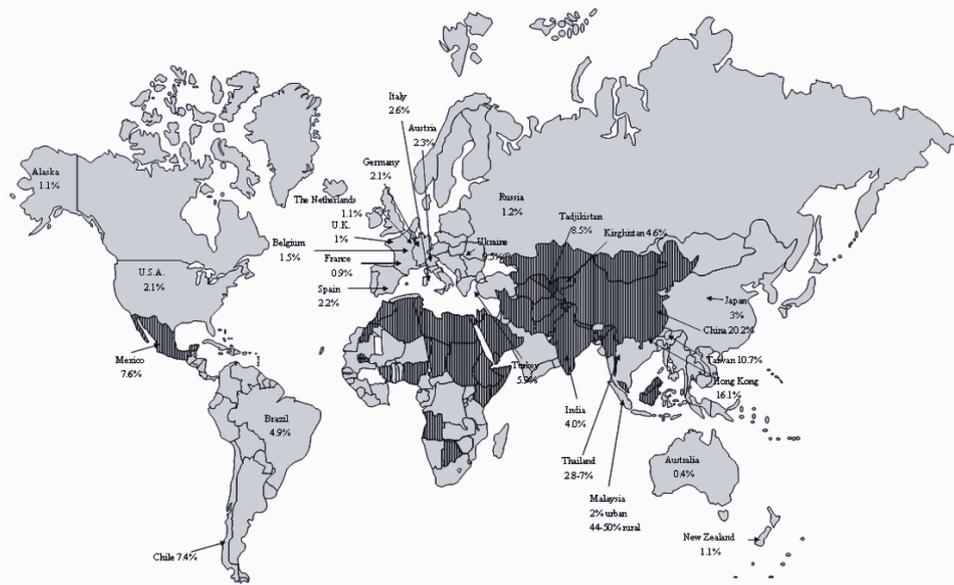
Figure 2. Global seroprevalence of anti-HEV antibodies [Adapted from ref. 177]. Shaded areas indicate that >25% of sporadic non-ABC cases are caused by HEV.

about 1% [182]. One of the seropositive individuals did not travel to a developing country in the months preceding blood donation, which suggests (a) local HEV source(s) in The Netherlands. This hypothesis was confirmed in 2003, when a cluster of three hepatitis E cases who had not traveled to a developing country during 3 months before onset of symptoms was observed [171]. All patients lived within 10 km from each other, which may suggest a common HEV-source. Despite retrospective interviews of the patients, however, no source was identified. A subsequent study of 209 acute non-ABC hepatitis cases from the same geographical area suggested that locally-acquired hepatitis E cases occurred more often in that region [165]. Hence, also in The Netherlands, one or more sources for HEV exist.

A number of epidemiological studies have focused on potential risk factors for HEV infection of humans by analyzing characteristics of hepatitis E patients that requested medical consultation in hospitals. Ijaz *et al.* [66] compared data from non-travel and travel associated hepatitis E in UK-patients. The authors 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 >65 years of age. Dalton *et al.* [24] and Mizuo *et al.* [109] both described a higher prevalence for males compared to females and for middle-aged or elderly patients. Mizuo *et al.* [109] 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.* [12] 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. In summary, recurring potential risk factors were especially gender and age, and possibly pre-existing diseases. These potential risk factors should be considered as risk factors for a severe form of hepatitis E that requires medical consultation (which relates to the design of the studies), but not as risk factors for HEV exposure.

Risk factors for HEV exposure are difficult to study considering the self-limiting or mild nature of infection in most cases, resulting in low numbers of identified infections. HEV has been detected an various animal species, including domestic pigs and wild boar [52], and these animal HEV strains can show high similarity to human HEV strains [106, 161]. Therefore, HEV is suggested to be a possible zoonotic virus, and human HEV-exposure may involve direct or indirect contact with HEV-infected animals.

1.3   Hepatitis E virus infection in pigs

Balayan *et al.* [5] reported in 1990 the possibility of HEV infection in pigs by experimentally infecting pigs with HEV obtained from a human patient (the HEV genotype was unknown). The aim of his experiment was to assess whether or not HEV can replicate in vertebrates other than primates, which had been described at that time. In 1995, Clayson *et al.* [21] observed HEV in domestic pigs in Nepal, and raised concerns about zoonotic transmission of HEV in developed countries. In 1997, Meng *et al.* [106] showed that HEV was 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 [2, 22, 185].

As shown 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, but this transmission has not been examined to date. In theory, the high prevalence of HEV among pigs may also be caused by continuous infection from other HEV sources. Therefore, it is important to assess whether pigs are a core or satellite population for HEV. In a core population infection is maintained without interference of other HEV-sources, whereas in a satellite population other sources of HEV are required continuously. The distinction between core and satellite populations can be based on the basic reproduction ratio R_0, which defines the average total number of new infections caused by one infectious individual in a completely susceptible population during its entire infectious period [1, 100]. If R_0 exceeds unity significantly, then pigs are a core population for HEV [31]. Whether R_0 for HEV transmission among pigs exceeds unity, however, is unknown.

Infections by HEV of genotype 3 in pigs cause in general no clinical symptoms [77, 79]. Jaundice in pigs was reported after experimental infection with HEV in a single experiment [5], but this observation is not confirmed by others. Subclinical symptoms in naturally infected pigs can be mild or moderate hepatic lesions [87, 95]. HEV infection does not affect litter size, an important production parameter for breeding sows [79]. The effects of HEV infection on weight gain and feed conversion (two other important production parameters for fattening pigs) have not been investigated to date.

Pigs may become infected early in life, as two-week-old piglets were observed to excrete HEV faecally [46, 85]. However, the highest prevalence of fecal HEV excretion is observed

Table 1. Prevalence and genotype of HEV RNA detected in pig samples globally.

Country	Year of sampling	Sample type	No. of pigs	% pigs pos.	No. of farms	% farms pos.	No. of sequences	Genotype (n)[1]	Ref.
Asia									
China	n.a.[2]	Serum	263	1.9	n.a.	n.a.	5	4	[166]
	2002-2004	Faeces	282	9.6	n.a.	n.a.	10	4	[185]
	2002-2004	Bile	160	3.1	n.a.	n.a.			[185]
India	2000	Serum	284	4.6	n.a.	n.a.	12	4	[2]
	1985-1987	Serum	45	4.4	n.a.	n.a.	2	4	[3]
	1999	Serum	12	33.3	n.a.	n.a.	4	4	[3]
	n.a.	Faeces (slaughter)	210	0.5	n.a.	n.a.	1	4	[142]
	n.a.	Faeces	94	0	1	-	-	-	[142]
Indonesia	2003	Serum	99	1.0	8	12.5	1	4	[168]
	2004	Serum	101	5.0	n.a.	n.a.	5	4	[169]
Japan	n.a.	Faeces	386	22.3	3	100	26	3	[111]
	n.a.	Faeces	186	1.6	12	25	3	3	[112]
	2000-2002	Serum	1360	13.7	25	88	137	3 (128), 4 (9)	[147]
	2001-2002	Serum	1425	3.9	92	34	55	3 (52), 4 (3)	[148]
	2002-2004	Serum	152	13.8	3	66.7	22	3	[150]
Korea	n.a.	Serum	128	2.3	10	n.a.	3	3	[20]
	1995-2004	Hepatic tissue	388	10.8	388	10.8	42	3	[72]
Mongolia	2006	Serum	243	36.6	4	100	89	3	[90]
Taiwan	n.a.	Serum	56	1.8	2	50	1	4	[59]
	n.a.	Serum	235	1.3	n.a.	n.a.	3	4	[178]
	1998-2000	Serum	521	1.5	n.a.	n.a.	4	3 (3), 4 (1)	[179]
	1998-2000	Faeces	54	5.6	n.a.	n.a.			[179]
Thailand	n.a.	Serum	76	13.2	4	25	10	3	[22]

[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	[161]
	2005	Faeces (pooled)	n.a.	n.a.	97	54.6	38	3	[128]
Spain	2003-2004	Various[3]	69	37.7	23	n.a.	26	3	[27]
	2002-2004	Faeces	146	23.3	21	38	9	3	[46]
	2002-2004	Faeces (pooled)	n.a.	n.a.	16	50	n.a.	n.a.	[46]
	>2001	Faeces	41	17.1	1	-	n.a.	3	[139]
	>2001	Serum	66	27.3	1	-			[139]
UK	n.a.	Faeces	40	22.5	1	-	2	3	[8]
North America									
USA	n.a.[2]	Faeces	80	40.0	29	65.5	} 27	3	[62]
	n.a.	Serum	16	12.5	8	12.5			[62]
	2002	Faeces (pooled)	n.a.	n.a.	28	25	7	3	[78]
South America									
Argentina	n.a.	Faeces	54	88.9	1	-	7	3	[110]
Mexico	n.a.	Serum	125	6.4	10	30	7	3	[22]
	n.a.	Faeces	90	31.1	9	56	21	3	[22]
Oceania									
New Zealand	n.a.	Faeces	45	37.8	2	n.a.	7	3	[48]

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

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	[103]
	n.a.	IgG	419	78.8	n.a.	n.a.	[166]
India	1985-1987	IgG	45	93.3	n.a.	n.a.	[3]
	1988	IgG	137	74.4	n.a.	n.a.	[4]
	1993	IgG	97	54.6	n.a.	n.a.	[4]
	1999	IgG	12	100	n.a.	n.a.	[3]
	2000	IgG	284	42.9	n.a.	n.a.	[2]
Indonesia	2003	IgG	99	71.7	8	100	[168]
Japan	.	IgG	107	39.3	3	100	[111]
	2000-2002	IgG	2500	57.9	25	100	[147]
	2001-2002	IgG	1425	55.7	n.a.	n.a.	[148]
	2001-2002	IgM	1425	7.0	n.a.	n.a.	[148]
	2001-2002	IgA	1425	11.7	n.a.	n.a.	[148]
	2002-2004	IgG	152	13.0	n.a.	n.a.	[150]
Korea	n.a.	IgG	264	14.8	13	85	[20]
	n.a.	IgG	140	40.7	n.a.	n.a.	[103]
Lao	1998	IgG	301	15.3	n.a.[1]	46	[10]
	2001	IgG	586	51.2	n.a.	n.a.	[10]
Mongolia	2006	IgG	243	91.8	4	100	[90]
Taiwan	n.a.	IgG	275	37.1	10	90	[59]
Thailand	n.a.	IgG	75	30.7	4	75	[103]

[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	[19]
New Zealand	n.a.	IgG	72	75.0	22	91	[48]
Europe							
Netherlands	n.a.[1]	IgG	34	23.5	n.a.	n.a.	[8]
Spain	1998-2000	IgG	439	41.9	41	98	[139]
	1998-2000	IgM	418	28.2	41	83	[139]
Sweden	n.a.	IgG	204	58.0	n.a.	n.a.	[8]
UK	1991-2001	IgG	256	85.5	n.a.	n.a.	[8]
North America							
Canada	n.a.[1]	IgG	712	18.1	67	55	[103]
Canada	1998-2000	IgG	998	59.5	n.a.	n.a.	[181]
USA	n.a.	IgG	84	34.5	4	100	[175]
USA	n.a.	IgG	283	71.4	15	100	[106]
South America							
Argentina	n.a.	IgG	97	22.7	5	100	[110]
Brazil	n.a.	IgG	427	57.1	n.a.	n.a.	[164]
Mexico	n.a.	IgG	125	80.0	10	30	[22]

1 n.a.: not available

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among pigs from 10 weeks of age up until 3 months [111, 85, 139]. This 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 [147, 148].

Porcine HEV strains of genotype 3 can cause infection in primates and human HEV strains of genotype 3 can cause infection in pigs [105]. These data further suggest the possibility of zoonotic transmission of 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 HEV exposure, as elevated HEV seroprevalence were observed in swine farm workers and veterinarians [59, 36, 107, 175]. Also foodborne transmission of swine HEV is proposed, because porcine liver from retail may contain HEV RNA [180] and infectious HEV [43]. 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. [153].

For the Netherlands, the possibility of HEV transmission from domestic pigs to humans is currently not investigated. With the high number of pigs in The Netherlands that are raised annually, the pool of swine HEV can be extensive. From a public health perspective, an assessment of the implications of HEV in swine for human HEV-exposure is warranted.

1.4   Aim and outline of this thesis

The project described in this thesis studied in detail pigs as host for HEV and made a first assessment of potential human exposure to swine HEV. First, the transmission potential of HEV among pigs was assessed to determine whether pigs can be a true animal reservoir for HEV (core population) or are victim to infection from another source (satellite population). To this end, we estimated whether the R_0 for HEV transmission among domestic pigs exceeds unity (Chapter 2). Furthermore, to identify the potential transmission routes, the course of infection in contact-infected pigs was studied, including the identification of ex- and secreta as possible sources for HEV-transmission and the period of infection during which these sources can contribute to transmission (Chapter 3). The time until and duration of viremia and faecal HEV excretion, time until seroconversion, the course of liver enzyme levels in serum and localization of HEV in the pig are examined. In Chapter 4, these and literature data are used in a Monte Carlo simulation model to estimate the probability of infection through faecal-oral transmission of HEV among pigs. For pigs to be a possible source of human HEV infections, humans should also be exposed to porcine HEV. Therefore, two possible routes of human exposure to porcine HEV are studied: foodborne

transmission by consumption of porcine livers (Chapter 5), and transmission due to direct contact with pigs (Chapter 6). In the final chapter, Chapter 7, the results will be discussed jointly.

Chapter 2

Estimation of hepatitis E virus transmission among pigs due to contact-exposure

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ABSTRACT

Locally acquired hepatitis E in humans from industrialized countries has been repeatedly suggested to originate from pigs. Pigs may serve as a reservoir of hepatitis E virus (HEV) for humans when a typical infected pig causes on average more than one newly infected pig, a property that is expressed by the basic reproduction ratio R_0. In this study, R_0 for HEV transmission among pigs was estimated from chains of one-to-one transmission experiments in two blocks of five chains each. Per chain, susceptible first-generation contact pigs were contact-exposed to intravenously inoculated pigs, subsequently susceptible second-generation contact pigs were contact-exposed to infected first-generation contact pigs, and lastly, susceptible third-generation contact pigs were contact-exposed to infected second-generation contact pigs. Thus, in the second and third link of the chain, HEV-transmission due to contact with a contact-infected pig was observed. Transmission of HEV was monitored by reverse transcriptase polymerase chain reaction (RT-PCR) on individual faecal samples taken every two/three days. For susceptible pigs, the average period between exposure to an infectious pig and HEV excretion was six days (standard deviation: 4). The length of HEV-excretion (*i.e.* infectious period) was estimated at 49 days (95% confidence interval (CI): 17 to 141) for block 1 and 13 days (95% CI: 11 to 17) for block 2. The R_0 for contact-exposure was estimated to be 8.8 (95% CI: 4 to 19), showing the potential of HEV to cause epidemics in populations of pigs.

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Introduction

Hepatitis E virus (HEV) is a cause of hepatitis among humans predominantly in developing countries. However, more and more HEV infections are being identified in industrialized countries. Partly, these infections result from travel to HEV-endemic areas; for the other part, the sources of HEV are mostly unknown [171, 66]. Several animal species have been suggested as possible sources, including cattle, rodents, dogs, wild boar, deer, horses and domestic pigs [106, 4, 161, 146, 131]. Amongst these species, domestic pigs carry HEV most ubiquitously, with prevalence estimates of more than 50% for both the pig- and farm-level [103, 147, 148, 128].

Because HEV strains from domestic pigs and humans show extensive similarity, zoonotic transmission has been suggested [106, 117, 57]. This suggestion is supported by successful experimental infection of pigs with human HEV and nonhuman primates with porcine HEV [105]. Furthermore, exposure to domestic pigs was identified by serological studies as a potential causal factor for human cases of hepatitis E [36, 107, 13].

If human cases of hepatitis E are caused by exposure to pigs, then at least HEV should persist and be transmitted within populations of pigs. In other words, pigs should be a true animal reservoir for HEV, defined as a population of animals that can maintain HEV infection without the need of other HEV sources. To assess whether pigs are a true animal reservoir, two aspects need to be studied. First, the infected pigs should be able to transmit HEV to other pigs. Second, the contact structure within farms should allow persistence of HEV. Although the high level of HEV infection in domestic pigs strongly suggests that pigs meet these two requirements, crucial evidence to support this suggestion is lacking. Evidence can be provided by quantifying the basic reproduction ratio (R_0), which defines the average total number of new infections caused by one typical infectious animal during its entire infectious period in a completely susceptible population [1, 100]. When R_0 is larger than 1, HEV transmission can be maintained among pigs. Estimates and confidence intervals for R_0 can be obtained by transmission experiments [31].

Published animal infection experiments with HEV have mainly used intravenous inoculation as the route of infection, but intrahepatic inoculation is also described [63]. The intravenous route is more efficient in causing HEV infection in pigs and primates than the oral route [121, 76]. This higher efficiency is needed when one needs to be certain that HEV, when present, causes infection. However, the natural route for HEV-transmission is most likely faecal-oral [17, 125]. The natural route of transmission is required to study transmission dynamics and dynamics of HEV infection in individual animals. Furthermore, infection due to contact-exposure has been observed previously [105, 76]. Therefore, studies

on HEV dynamics in pigs are ideally done with contact-infected pigs, using exposure of susceptible pigs to infectious pigs.

The objective of the current study was to quantify the transmission of HEV among pigs that were infected by exposure to an infectious pig. Therefore, a transmission chain was designed: susceptible first-generation contact pigs were contact-exposed to intravenously inoculated pigs; susceptible second-generation contact pigs were contact-exposed to infected first-generation contact pigs; and susceptible third-generation contact pigs were contact-exposed to infected second-generation contact pigs. Data from the transmission chains were used to estimate the period between infection and HEV excretion, the number of days of HEV excretion (*i.e.* infectious period) and ultimately R_0.

2.2   Materials and Methods

2.2.1   Stable design

Power-calculations demonstrated that ten replicates of a transmission chain were needed, each replicate requiring a separate stable to avoid HEV-transmission between replicates. The stables were subdivided in three adjacent compartments of about 3 m2 each, with a main passage in front for access and waste removal (Figure 1). All inner walls in the stable were made of new multiplex wood of 145 cm in height. The walls that separated compartments contained plastic windows to enable visual contact between pigs in neighboring compartments (required by the ethical committee). Within compartments, all joints between walls and the floor were sealed. The floors of the stables were solid and a thin layer of saw dust as bedding was supplied (required by the ethical committee). Four male pigs of 3–4 weeks of age were allotted to one stable one week before inoculation. All pigs were of about the same age at the start of the experiment. Two pigs were placed in the compartment furthest from the main entrance of the stable (compartment 1); each of the other two pigs was placed in one of the two remaining compartments. Faeces of pigs tested negative for the presence of HEV RNA at 14 days and 7 days before inoculation. Furthermore, serum samples collected at 7 or 4 days before inoculation tested negative for anti-HEV antibodies, indicating an absence of maternal immunity. Therefore, pigs were considered susceptible for HEV-infection. Since only five stables were available simultaneously, the experiment was done in two separate blocks, using five stables per block. After the first block and before the second block, all stables were thoroughly cleaned with water containing hypochlorite, disinfected by fumigation with formaline, cleaned with water under high pressure and treated consecutively with acid, sodiumhydroxide and a detergent. Swab samples were taken from the walls, the floors, the drinking nipples, the boots, the, the disinfection tub, the
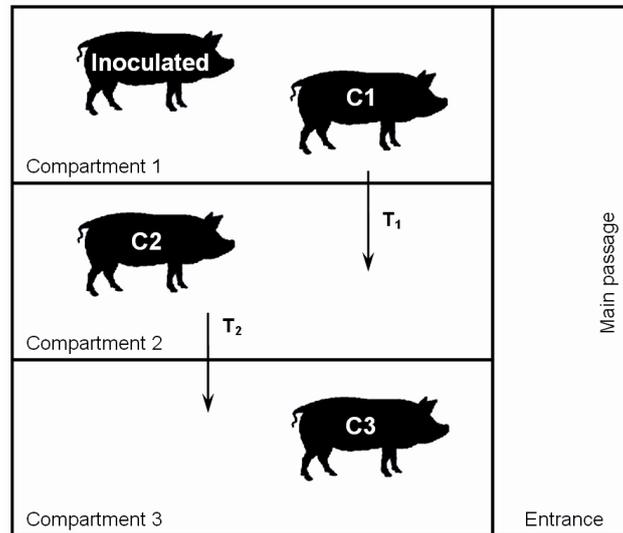
Figure 1. Schematic representation of the experimental setup to study transmission of hepatitis E virus in pigs. Intravenously inoculated pigs were to infect first-generation contact pigs (C1). Once infected, C1-pigs were transferred to the second compartment (at time T1) to infect second-generation contact pigs (C2). Once infected, C2-pigs were transferred to the third compartment (at time T2) to infect third-generation contact pigs (C3). Data on transmission from C1- to C2-pigs and from C2- to C3-pigs were used to estimate R_0 for hepatitis E virus.

ventilator and the manure pit, and were shown to be free of HEV RNA and therefore presumably HEV particles before the start of the second block.

This animal experiment was approved by the Ethics Committee on Animal Experiments of the Animal Sciences Group of Wageningen University and Research in Lelystad. The experiment was done in a BSL-2 facility.

2.2.2 Virus

HEV was isolated from a Dutch finishing pig and was characterized as genotype 3 (GenBank: DQ996399). To obtain sufficient virus for the experiment, the virus was amplified by intravenous inoculation in a caesarean-derived, colostrums deprived pig under high health conditions. The infected pig was euthanized 28 days post infection (dpi) and the liver was used to prepare inoculates. The pig tested negative for porcine reproductive and respiratory syndrome virus, porcine circovirus type 2 and enterovirus by reverse transcriptase polymerase chain reaction (RT-PCR).

To prepare the inoculates, the liver was grinded in EMEM (Gibco®, Invitrogen™, Breda, The Netherlands) with a mortar and pestle. The suspension was transferred to a 15 ml tube. After 15 min of settlement, the supernatant was transferred to a new tube and filtered consecutively through microfilters of 0.45 μm and 0.22 μm pore size. The suspension was aliquoted in volumes of 2.5 ml and stored at −70ºC.

The inoculates contained about 10^4 PCR detectable units of HEV RNA per ml, quantified as the most probable number using maximum likelihood estimates based on the end-point dilution in RT-PCR [159]. Each intravenously inoculated pig received 2 ml of inoculum in the first block and 1 ml of inoculum in the second block, resulting in a 0.3 $\log_{10}$ difference in HEV titer between the blocks. The inoculates were administered via the *vena auricularis*.

2.2.3 Transmission chains

In total, ten replicate transmission chains were done. Each transmission chain consisted of three one-to-one exposures, *i.e.* one susceptible pig was contact-exposed to one infectious pig. The infectious pigs for the first one-to-one exposure were created by intravenous inoculation of HEV (*iv*-pigs). After inoculation, the first-generation contact pigs (C1) were contact-exposed to *iv*-pigs (Figure 1). When C1-pigs excreted HEV RNA in faeces at three consecutive samplings, the pigs were assumed to be infected and infectious, and were transferred to the adjacent compartment with a second-generation contact pig (C2). When the C2-pigs excreted HEV RNA in faeces at three consecutive samplings, they were transferred to the adjacent compartment with a third-generation contact pig (C3). When a C3-pig excreted HEV RNA in faeces at three consecutive samplings, the transmission chain ended. Transmission chains also ended when an infectious pig stopped to excrete HEV in the faeces before infecting the contact pig. Pigs were euthanized after successful transmission of HEV to contact pigs, or when the transmission chain in that stable ended.

2.2.4 Sample collection

Individual faecal samples were collected from each pig in the transmission chain at seven days before infection and the day of infection. Upon inoculation on a Monday, animal-technicians collected individual faecal samples from the pigs in one-to-one experiments every Monday, Wednesday and Friday. When contact-exposed susceptible pigs were positive for HEV RNA in faeces at two consecutive samplings, a control sample was taken from the next-generation contact pig to examine the absence of HEV RNA.

In each stable, samples were taken from the susceptible contact pig first, followed by the infectious pig. When a control sample from a next-generation contact pig was required, this

sample was taken before the other samples. In each stable and at each sampling, new disposable overalls, gloves and facial masks were used. Furthermore, a pair of boots was present in each stable, which were cleaned and disinfected after each sampling. These measures should minimize the risk of transmission of HEV by the animal technicians. Faecal samples were cooled on ice, transported to the laboratory, and processed immediately. Samples were mixed 1:1 in tryptone soya broth with 20% glycerol. About 200 mg of this 1:1-solution was further diluted 1:5 to obtain a 10% suspension in Hank's balanced salt solution containing 50 μg/ml gentamycine; the remainder was stored at −70ºC. In total, 140 μl of the 10% suspension was used to extract RNA with the QIAamp Viral RNA Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer. RNA was used immediately for HEV RT-PCR amplification.

2.2.5 HEV RT-PCR and Southern blot hybridization

For the detection of HEV RNA, a single round RT-PCR with primers ORF2-s1 and ORF2-a1 was used [138]. This RT-PCR yielded a fragment of 197 nucleotides. The RT-PCR fragment was separated in a 2% agarose gel and HEV RT-PCR fragments were confirmed by Southern blot hybridization [161]. To monitor possible inhibition during RT-PCR, an internal RNA control and a ten-fold serial dilution of undiluted sample RNA were included at the stage of reverse transcription [128]. Three serial tenfold-dilutions of porcine HEV RNA of genotype 3 (GenBank: DQ996399) were used as the positive RT-PCR control. The second serial dilution of control RNA contained a concentration around the detection limit of the RT-PCR. RNase free water was used as the negative control.

2.2.6 Transmission model

Transitions of pigs from the susceptible state (S) to the infectious state (I), and from the infectious state to the recovered state (R) were described by a stochastic SIR-model [e.g. 9, 34]. Pigs were considered susceptible when antibodies against HEV were absent and considered infectious when HEV was excreted faecally. Transitions from S to I occur with transmission rate β, defined as the average number of successful transmissions by an infectious individual per day in a completely susceptible population (where $S / (S + I + R) \approx$ 1). A modification was made to the SIR-model proposed by Becker [9] by introducing an effect of population size on β (frequency dependent transmission model), because this was shown to approximate reality more closely [28, 30].

Transitions from I to R in the SIR-model depend on rate of recovery α, defined as the reciprocal of the infectious period in days. Since β/α gives the estimate for R_0, rate of transmission and length of the infectious period need to be estimated.

2.2.7 Estimation of the rate of transmission

Use of one-to-one challenges to quantify transmission of a pathogen has the advantage that the occurrence of transmission becomes a binomial parameter (transmission does occur or not). To estimate β, each pig was allocated to one of the states *S* or *I* at each sampling based on the absence (*S*) or presence (*I*) of HEV RNA in faeces. However, due to a delay between actual infection and first HEV-excretion in faeces–the latent period–pigs may already be infected without excreting HEV. Therefore, dpi of first HEV excretion in faeces should be adjusted to the actual moment of infection by subtracting the length of the latent period. Since data on the length of the latent period for HEV in pigs are lacking, we estimated the latent period from data on *iv*-pigs, where the moment of infection was known. To this end, dpi for the last negative faecal sample and the first positive faecal sample were averaged per *iv*-pig. The mean of these averages was the estimate for the latent period. If adjustment for the latent period subsequently yielded an estimated moment of infection prior to exposure to an infectious pig, then the moment of infection was set to the first sampling-interval in which contact-exposure occurred. Since next-generation contact pigs in our experiments sporadically excreted HEV before being contact-exposed to an infectious pig, we needed to consider the transmission between compartments in addition to the transmission within compartments. To estimate both transmission rates, β was divided in rate of transmission within compartments (β_w) and an additional rate of transmission for transmission between compartments (β_a) [cf. 82]. The rate of transmission between compartments (β_b) was the sum of β_w and β_a. For each susceptible pig, infectious pigs were counted in the own (I_w) and in neighboring (I_b) compartments, with I_{tot} being (I_w+I_b). Furthermore, the fraction of all the infectious pigs that was present in the neighboring compartment (I_b/I_{tot}) was represented by *f*. The probability of infection per unit of time (*p*) can now be described as a function of β, *I*, *N*, and time interval between samplings (Δt):

$$p = 1 - e^{-\left(\beta_w + \beta_a^f\right)\frac{I_{tot}}{N}\Delta t}$$

(1)

The value of *p* is estimated by the expected number of new cases (E(C)) divided by the number of trials in a binomial process (in our situation *S*). To obtain linearity in regression parameters, a complementary log-log link function can be applied:

$$c \log \log\left[\frac{E(C)}{S}\right] = \log\left(\beta_w\right) + f \log\left(\beta_a\right) + \log\left(\frac{I_{tot}}{N}\Delta t\right)$$

(2)

This model was analyzed statistically with STATA 9 (StataCorp LP, College Station, USA) as a generalized linear model with the complementary log-log link function, the term $\log(I_{tot}/N \times \Delta t)$ as offset, f as explanatory variable and S as the number of trials in the binomial process.

### 2.2.8 Estimation of the length of the infectious period

Because R_0 represents the number of new infections caused by an infected pig during the entire infectious period, the length of the infectious period needs to be estimated. The infectious period was defined as the average number of days that infected pigs excreted HEV, and was estimated with survival analysis [81] from data on contact-infected pigs. Because of the two- or three-day intervals between consecutive samplings, the start of HEV-excretion was calculated as the average of dpi of the last negative and first positive sampling. Analogously, the end of HEV excretion was calculated as the average dpi between last positive and first negative samplings.

2.3 Results

2.3.1 HEV excretion and transmission

All ten *iv*-pigs started to excrete HEV in faeces between 0 dpi and 7 dpi (Figure 2). The average period between HEV inoculation and start of HEV excretion–the latent period–was three days (range: 2 to 7, median: 3). The latent period did not differ significantly between block 1 and block 2 ($P_{Kruskal-Wallis}$=0.40). All ten *iv*-pigs transmitted HEV to C1-pigs (Table 1). For C1-pigs, periods between exposure to infectious *iv*-pigs and HEV excretion did not differ significantly between block 1 (9 days) and block 2 (17 days) ($P_{Kruskal-Wallis}$=0.09). The C1-pigs that shed HEV in faeces at three consecutive samplings were transferred to compartments with C2-pigs and HEV excretion was monitored (Figure 2). HEV transmission occurred to four C2-pigs in block 1 and to three C2-pigs in block 2. In block 1, one C2-pig excreted HEV before being contact-exposed to a C1-pig. Nevertheless, the transmission chain was continued by contact exposing a C3-pig to this C2-pig. For C2-pigs, periods between exposure to infectious C1-pigs and HEV-excretion did not differ significantly between block 1 (three days) and block 2 (nine days) ($P_{Kruskal-Wallis}$=0.10).

The C2-pigs that shed HEV in faeces at three consecutive samplings were transferred to compartments with C3-pigs and HEV excretion was monitored in both pigs. HEV was transmitted to five C3-pigs in block 1. In block 2, two C3-pigs excreted HEV before being contact-exposed to a C2-pig. The other C3-pig in block 2 did not excrete HEV in faeces. The average period between exposure and first HEV excretion by the C3-pigs was six days.
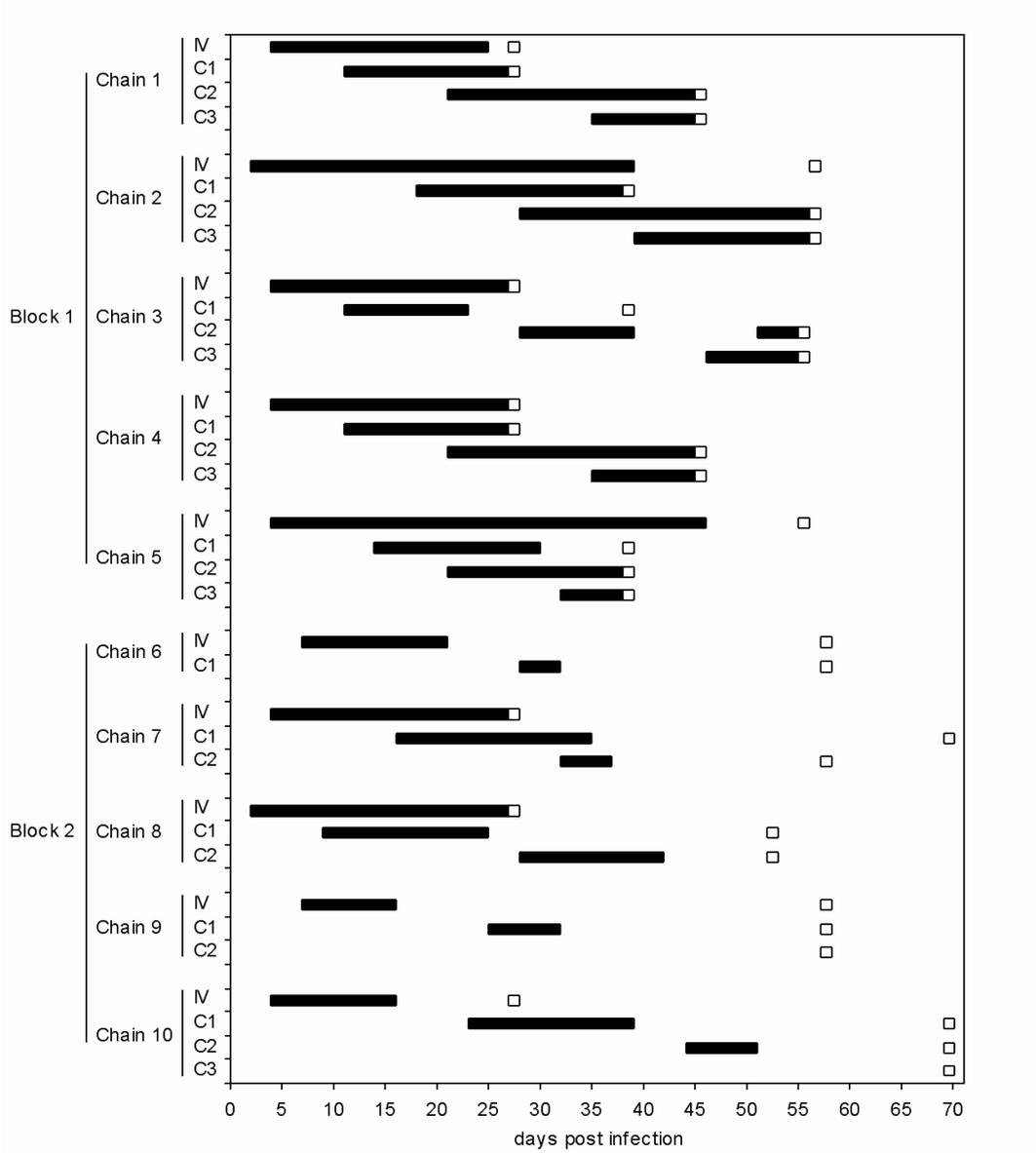
Figure 2. HEV RNA excretion in faeces per pig (*iv*-C3) for the two blocks. Each set of four, three or two pigs represents a transmission chain. For details on transmission chains, see Figure 1. The euthanization of pigs is represented by '□'.

Table 1. Transmission of hepatitis E virus by contact-exposure of susceptible pigs to infectious pigs.

	Transmission from[a]					
	iv-pigs to C1-pigs		C1-pigs to C2-pigs		C2-pigs to C3-pigs	
	Success	t^b **(SD)**	**Success**	t **(SD)**	**Success**	t **(SD)**
Block 1	5/5	9 (4)	4/4[c]	3 (4)	5/5	6 (3)
Block 2	5/5[d]	17 (7)	3/4	9 (4)	0/1[e]	.
Total	10/10	13 (7)	7/8	6 (4)	5/6	6 (3)

[a] *iv*: intravenously inoculated; C1: first-generation contact pig; C2: second-generation contact pig; C3: third-generation contact pig.

[b] Average number of days between exposure and estimated moment of infection.

[c] One C2-pig excreted HEV before being contact-exposed to the C1-pig.

[d] One C1-pig excreted HEV in faeces at three consecutive samplings, but HEV excretion was ended at day of transfer to the next compartment.

[e] Two C3-pigs excreted HEV before being contact-exposed to the C2-pig.

2.3.2   Quantification of HEV transmission

The latent period for *iv*-pigs of three days was used to adjust the moment of becoming infectious to the actual moment of infection. This resulted in overall maximum likelihood estimates for the rate of transmission within compartments (β_w) of 0.66 (95% CI: 0.32 to 1.35) per day, and for the rate of transmission between compartments (β_b) of 0.02 (95% CI: 0.01 to 0.04) per day. There was no observed difference in estimates for β_w and β_b between the two blocks, as determined from the difference in maximum likelihood between the models with and without 'block' as the explanatory variable ($P_{chi-square}$=0.21). The period of HEV excretion was observed for ten of 23 pigs; for the other 13 the endpoint of HEV excretion in faeces was not determined (*i.e.* data were censored). Subsequently, the infectious period was estimated at 49 days (95% CI: 17 to 141) in block 1 and at 13 days (95% CI: 11 to 17) in block 2. The difference between the two blocks was statistically significant (*P*<0.01). Since the main objective of the current study was to assess whether or not HEV is transmitted among pigs, we were interested in whether or not "1" is excluded from the 95% confidence interval of R_0. By using the estimate of 13 days for the infectious period, we tested the hypothesis that HEV transmission does not occur among pigs conservatively. Since R_0 is the product of β_w and the length of the infectious period, this mounts to 8.8 (95% CI: 4.2 to 18.8). When the infectious period of 49 days was used to estimate R_0, then R_0 equalled 32 (95% CI: 11.2 to 92).

2.4   Discussion

Experimental HEV-infection in susceptible pigs has been established through intravenous, oral and intrahepatic inoculation [5, 76, 63] and through contact-exposure to an infectious pig [105, 76]. Intravenous inoculation is used most often, since it has a higher probability of success compared to oral inoculation [77]. It is, however, not the natural route of transmission, which is supposedly faecal-oral transmission. In the current study, we used

intravenous inoculation to obtain infectious pigs. Subsequently, we used the intravenously inoculated pigs to generate first-, second and third-generation contact-infected pigs to estimate the R_0 for transmission of HEV in pigs. The R_0 was estimated as 8.8 and was significantly larger than 1, which indicates that HEV is able to spread when contacts between pigs occur randomly [29]. Therefore, pigs can be a true animal reservoir for HEV. Whether the status of true animal reservoir applies to field conditions (*i.e.* pig farms) as well, however, remains to be assessed. Field and experimental conditions differ, amongst others, in the contact-structure between pigs. The contact-structure at the farm-level can be defined at different levels: multiple pigs represent a pen, multiple pens represent a unit, and multiple units represent a barn. The frequency and intensity of contact between pigs at different levels of the contact-structure is different than that for pigs within the same level. This will likely lower the R_0 on pig farms as compared to the R_0 in the current experiment.

Recently, Satou and Nishiura [134] used cross-sectional serological data from pigs of different ages to estimate the force of infection for HEV. Using that force of infection estimate, R_0 was estimated at 4.02–5.17. By calculating R_0 from the estimated force of infection it was assumed that new infections in pigs were solely caused by infectious pigs. Based on our results, showing that HEV is indeed transmitted among pigs, this is in retrospect probably a correct assumption. The estimates by Satou and Nishiura [134] are about half our estimate of R_0. This difference may be due to differences in contact structure between pigs on pig farms and the pigs in our experiment. Furthermore, a different definition of HEV infection, *viz* based on seroconversion or virus excretion, may also contribute to different R_0 estimates. The sensitivity and specificity of the assays used to detect either anti-HEV antibodies or HEV genomes will differ. Furthermore, seroconversion is not detected in all pigs that excrete HEV faecally [77, 76]. In addition to a false-negative test result, this finding may also indicate the absence of antibody development in some pigs. These pigs will not be considered infected when serological data is used as the definition of infection. Therefore, differences in the R_0-estimates between the current study and the study by Satou and Nishiura [134] are expected.

On three occasions, contact-pigs excreted HEV in faeces before being contact-exposed to an infectious pig. One pig showed HEV RNA and anti-HEV antibodies also in faeces and serum, respectively, taken two or three days pre-exposure; the other two pigs did not (data not shown). Since anti-HEV antibody can be detected in serum at the earliest two weeks after intravenous inoculation [54], the seroconverted pig was infected likely more than two weeks before taking the pre-exposure samples. The other two pigs were likely in the initial phase of infection, since only HEV RNA in faeces was detected. The design of compartments and the sequence of sampling of pigs were to minimize the risk of HEV transmission via direct contact between pigs from neighboring compartments or via animal technicians.

However, HEV transmission by these transmission routes cannot be excluded entirely. In addition, another possible route may be cross-over of HEV-contaminated bedding between compartments due to pig-activity. By including the possibility of HEV transmission from neighboring compartments in the statistical model, we estimated transmission within and between compartments. The transmission between compartments was estimated to be a factor 30 lower compared to transmission within compartments.

For three contact-infected pigs, the adjustment for the latent period would yield an estimated moment of infection within one day before introduction of the infectious pig. We, however, assumed that these pigs were contact-infected after introduction of the infectious pig, as permitted by the 95% confidence interval for the latent period. However, these pigs may also have been infected prior to contact-exposure. In that case, the rate of transmission between compartments would be increased at the expense of the rate of transmission within the compartment. Indeed, when assuming that these three pigs were infected prior to contact-exposure, the R_0 was estimated to be 7 (95% CI: 3.2 to 16.4) with the 95% confidence interval excluding "1". Therefore, our assumption does not affect the conclusion that HEV can spread among pigs.

Intravenously inoculated pigs in block 2 received a 0.3 $\log_{10}$ lower dose of HEV compared to the inoculated pigs in block 1. A dose-response relationship is described for HEV in pigs, with lower peak HEV-titers in faeces and a delay in first HEV-detection due to a three-log lower dose [105]. We observed no delay in first HEV-detection in faeces for inoculated pigs in block 2 compared to inoculated pigs in block 1, shown by the absence of a significant difference in the latent period between both blocks. This result was expected, since the difference in dose in our study was marginal compared to the three-$\log_{10}$ lower dose by Meng et al. [105]. In contrast, a shorter average infectious period for pigs from block 2 compared to pigs from block 1 was observed. Whether dose has an effect on duration of HEV-excretion is currently unknown, but we hypothesize that the different infectious periods are more likely the result of experimental variation (*e.g.* different genetic composition of pigs or a non-regulated relative humidity) than of the marginal different doses.

The period between exposure of susceptible pigs to infectious pigs and the subsequent HEV-excretion differed for C1-pigs (13 days), and C2 and C3-pigs (6 days). The C1-pigs were exposed to *iv*-pigs from inoculation onwards. In contrast, the C2-pigs and C3-pigs were exposed to infectious pigs that had already excreted HEV at the last three samplings. Concentrations of HEV RNA in faeces for a third positive faecal sample were generally higher than for the first positive faecal sample (data not shown). A higher concentration of HEV in faeces may increase the probability of HEV-transmission per unit of time compared

to a lower concentration, assuming that the amount of faecal excretion is not negatively correlated with HEV concentration in faeces. A higher probability of HEV-transmission will on average shorten the period between exposure and infection. This shorter period can be explained in binomial terms by an increased probability of success. An increased probability indicates that the average number of successful transmissions per day (β) will be higher, which results in a higher estimate for R_0. In this perspective, our estimate can be deemed conservative. The current estimate of R_0 for HEV was based on a single subtype within genotype 3. Possible strain-dependent infectivity of HEV-subtypes may influence the time period between exposure and infection. Different subtypes of genotype 1 strains of HEV, for instance, infected different numbers of HEV-permissive cells in culture, suggesting differences in infectivity between subtypes within one genotype [38]. Different infectivity will have an effect on transmission of a pathogen, since these differences are related directly to differences in probability of infection per HEV particle [154]. Therefore, a different probability of infection per HEV particle will alter the length of the period between exposure and infection and hence the estimate for β and R_0. Since many different subtypes of HEV are present among pigs and humans in industrialized countries [91], differences in virulence among different subtypes of HEV genotype 3 need to be assessed.

The period between actual infection and faecal HEV-excretion (in this study defined as the latent period) for HEV in pigs was estimated from data on intravenously inoculated pigs, because the actual moment of infection was known for these pigs. Oral intake of HEV, however, requires HEV to pass through the stomach and intestine and this may cause a delay in the start of the infectious period compared to intravenous inoculation [68]. However, by using the latent period for intravenously inoculated pigs, R_0 was estimated conservatively because a longer latent period results in an estimated actual moment of infection closer to the moment of first contact-exposure. This increases the probability of successful HEV-transmission per infectious pig per day and thereby β. Therefore, the conclusion that HEV is able to spread among susceptible pigs using the latent period for *iv*-pigs is conservative compared to the use of a longer latent period.

In conclusion, the current experimental design proved useful to study HEV transmission by contact-exposure. By doing so, we estimated R_0 at 8.8 (95% CI: 4.2 to 18.8), indicating that HEV is able to spread among pigs. Hence, pigs can be a true animal reservoir. Next, the reproduction ratio should be estimated for HEV transmission in pig-farms, where there is a different contact structure compared to the experimental conditions.

2.5 Acknowledgements

Chapter 3

The course of hepatitis E virus infection in pigs after contact-infection and intravenous inoculation

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ABSTRACT

Worldwide, hepatitis E virus (HEV) genotype 3 is observed in pigs and transmission to humans is implied. To be able to estimate public health risks from *e.g.* contact with pigs or consumption of pork products, the transmission routes and dynamics of infection should be identified. Hence, the course of HEV-infection in naturally infected pigs should be studied. To resemble natural transmission, 24 HEV-susceptible pigs were infected either by one-to-one exposure to intravenously inoculated pigs (C1-pigs; n=10), by one-to-one exposure to contact-infected pigs (C2-pigs: n=7; C3-pigs: n=5) or due to an unknown non-intravenous infection route (one C2-pig and one C3-pig). The course of HEV-infection for contact-infected pigs was characterized by: faecal HEV RNA excretion that started at day 7 (95% confidence interval: 5 to 10) postexposure and lasted 23 (19 to 28) days; viremia that started after 13 (8 to 17) days of faecal HEV RNA excretion and lasted 11 (8 to 13) days; antibody development that was detected after 13 (10 to 16) days of faecal HEV RNA excretion. The time until onset of faecal HEV RNA excretion and onset of viremia was significantly shorter for *iv*-pigs compared to contact-infected pigs, whereas the duration of faecal HEV RNA excretion was significantly longer. At 28 days postinfection HEV RNA was detected less frequently in organs of contact-infected pigs compared to iv-pigs. For contact-infected pigs, HEV RNA was detected in 20 of 39 muscle samples that were proxies for pork at retail and in 4 of 7 urine samples. The course of infection differed between infection routes, suggesting that contact-infection could be a better model for natural transmission than *iv* inoculation. Urine and meat were identified as possible HEV-sources for pig-to-pig and pig-to-human HEV transmission.

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3.1 Introduction

Hepatitis E virus (HEV) is a positive sense, non-enveloped single-stranded RNA virus with a genome of 7.2 kb and can be grouped into at least four genotypes [39]. Hepatitis E virus was considered to be restricted to developing countries, but it is now considered an emerging pathogen in developed countries [e.g. 132]. The epidemiology of HEV, however, differs between developed and developing countries [39]. In developing countries all four genotypes of HEV are found in locally acquired hepatitis E cases, whereas in developed countries locally acquired HEV cases are caused by genotypes 3 and 4 [91]. HEV infections with genotypes 1 and 2 are implicated in both epidemic and sporadic cases of HEV infection, whereas genotypes 3 and 4 have been only implicated in sporadic cases so far. Sources for these sporadic cases in industrialized countries are uncertain. The absence of person-to-person transmission of HEV genotype 3 among 18 household members of acute hepatitis E patients in the Netherlands [12] suggests that human HEV infections acquired in the Netherlands are of environmental origin rather than person-to-person transmission. Worldwide, HEV has been reported in environmental sources, including surface water [130], animal species including domestic pigs and wild boar [52], sewage of animal origin [117], and foods of animal origin [180, 152, 97, 15, 43]. Zoonotic foodborne HEV transmission via wild deer meat has been proven [153], but not from other environmental sources. An increased anti-HEV seroprevalence in people working professionally with pigs [175, 13] and presence of infectious HEV in commercial porcine livers at retail [43] however, suggests that swine may be a source of human exposure to HEV. Based on the phylogenetic similarity between HEV-sequences from human and swine, interspecies transmission was suggested [106, 57].

In the Netherlands, about 7.5×10^6 fattening pigs are raised annually [144]. HEV RNA was observed in faeces from >50% of Dutch fattening pig farms [128] and HEV has a high transmission potential among domestic swine [14]. Therefore, domestic swine may be an important reservoir for human HEV infections, but to which extent is currently unknown. To be able to estimate the public health risk using field data on the occurrence of HEV in pigs, the natural course of HEV infection in pigs needs to be known. Several studies have presented experimental data for intravenously (*iv*) inoculated pigs, showing onset of faecal HEV RNA excretion at one to two weeks postinoculation and onset of viremia at two to three weeks postinoculation [104, 105, 54, 79, 76]. Faecal HEV RNA excretion may last up to 7 weeks, whereas viremia is detected generally for one to three weeks [104, 76]. Faecal HEV RNA excretion is observed in all pigs after *iv* inoculation, but viremia and antibody development are not observed in all *iv* inoculated pigs. Antibodies to HEV infection are detected between two and eight weeks postinoculation [104, 105, 77]. Increased liver

enzyme levels in serum are generally not observed in *iv* inoculated pigs [104, 105, 54, 79].

However, whether *iv* inoculated pigs display a course of HEV infection that resembles that of naturally infected pigs (presumably via the faecal-oral route [39]) is currently unknown. Direct oral inoculation of HEV in pigs has been unsuccessful in all but one pig [76]. This pig received a dose of at least 10^8 HEV genome equivalents (one genome equivalent was defined as the number of HEV genomes present in the highest serial dilution positive by RT-PCR), whereas two other pigs that received this dose remained uninfected. Contact-exposure of a susceptible pig with an infectious pig appears to lead to HEV infection more easily than direct oral inoculation [104, 105, 76]. Thus, the use of contact-infected pigs may be a good alternative to study the course of HEV infection for naturally infected pigs. Contact-infected pigs are likely to reflect the natural course of HEV-infection more accurately than *iv* inoculated pigs.

The aim of the current study was to describe the course of HEV infection in contact-infected pigs by estimating the time until and the duration of faecal HEV RNA excretion and viremia and the time until antibody development. Localization of HEV in the pig and liver enzyme levels in serum were also assessed. As *iv* inoculated pigs were used to generate first-, second- and third-generation contact-infected pigs [14], the data for contact-infected pigs were compared to those for *iv* inoculated pigs to identify possible differences in the course of infection due to infection route.

3.2   Methods

3.2.1   Virus

Hepatitis E virus was acquired from a liver sample of a naturally infected Dutch fattening pig and handled as described previously (Chapter 2). The strain belonged to genotype 3.

3.2.2   Experimental design

The infection experiment is described in detail in Chapter 2. Briefly, this study was performed in two replicate blocks (blocks 1 and 2), each comprising 20 pigs of 3-4 weeks old at the start of the experiment. The HEV inoculum was prepared in bulk, aliquotted in 10 portions and stored at -70ºC until use. The pigs were obtained from a conventional, SPF herd, free of the most significant pig pathogens (PRRSV, Actinobacillus pleuropneumonia, M. pneumoniae). Pigs were tested to be HEV RNA negative in faeces at two weeks and one week prior to inoculation. Furthermore, the pigs tested negative for anti-HEV antibodies in serum one week prior to the start of the experiment and on the day the experiment started.

Blood samples were tested two weeks before start of the experiment to be negative for PCV2 and PRRSV by molecular methods.

Four pigs were allotted to each of five stables (~9 m² each) of a BSL2 facility, each stable containing three compartments. One pig was placed in each of two compartments and two pigs were placed in the third compartment. One of these two pigs was inoculated intravenously (iv-pig) with an estimated amount of ~10^4 PCR detectable units of HEV, while the other pig served as first-generation contact pig (C1-pig). Faecal samples were taken three times per week from the iv-pig and the C1-pig, and HEV excretion was monitored by conventional RT-PCR. When the C1-pig excreted HEV in faeces at three consecutive samplings it was moved to the compartment containing the second-generation contact pig (C2-pig). When the C2-pig excreted HEV in faeces at three consecutive samplings it was moved to the compartment containing the third-generation contact pig (C3-pig).

The experiment was approved by the institutes' animal ethical committee according to the Dutch law on animal experiments.

All 10 iv-pigs were HEV-infected and transmitted HEV to the C1-pigs by one-to-one exposure (Table 1). However, only eight of ten trials could be included in the analysis of HEV transmission from an infectious C1-pig to a C2-pig, because one infected C1-pig excreted HEV in faeces at three consecutive samplings, but then not from the day of transfer onwards, and faecal HEV RNA excretion was detected in one C2-pig prior to first exposure to an infected C1-pig (referred to as indirect contact infection). Seven of these C1-C2 trials were successful. However, because two C3-pigs were already non-contact infected and therefore out of the transmission analysis, and the indirectly infected C2-pig could be used for the analysis of HEV-transmission to a C3-pig, six trials were conducted to transmit HEV from a C2-pig to a C3-pig instead of seven. Of these six C2-C3 trials, five were successful. For two of the three indirect-contact infected pigs the onset of faecal HEV RNA excretion was known, and these pigs were therefore included in the current analyses. This yields a total of 24 pigs that were contact-infected (direct or indirect through an unknown route).

Table 1. Number of pigs that were HEV-infected due to infection routes other than intravenous inoculation (iv) in the experiment.

Type of pig	Direct transmission			Indirect transmission
	Transmission type	Exposed pigs	Infected pigs	
C1	$iv \rightarrow$ C1	10	10	0
C2	C1 $\rightarrow$ C2	8	7	1
C3	C2 $\rightarrow$ C3	6	5	2*
Number of pigs used in the analyses			22	2

* for one of the two pigs, the moment of becoming infected was unknown and could therefore not be used in the analyses.

3.2.3 Sampling

Faecal samples were taken and processed as described in Chapter 2. Serum samples were collected every three to four days (twice per week) and centrifuged at 2,500 g for 10 min to obtain serum. Part of the serum was stored at -20 ℃ for antibody detection at a later time, the remainder at -70 ℃ for RNA extraction at a later time. Blood samples were collected only from those pigs that were housed together in one compartment (*i.e.* where HEV transmission was analyzed). In addition, when a contact-infected pig showed HEV RNA excretion at two consecutive samplings, a control sample was taken from the next-generation contact pig. Faeces and serum were collected from two *iv*-pigs up until 56 dpi in Block 1 and from two C1-pigs up until 55 and 65 days after first faecal HEV RNA excretion in Block 2.

Five *iv*-pigs were necropsied at 28 dpi, five C2-pigs at 25 or 26 days after first faecal HEV RNA excretion (referred to as 28 days postinfection), two *iv*-pigs at 56 dpi in block 1 and two C1-pigs at 55 and 65 days after first faecal HEV RNA excretion in block 2. The five *iv*-pigs and C2-pigs (three each in block 1 and two each in block 2) were appointed by randomly selecting one of the five stables. The two *iv*-pigs necropsied at 56 dpi were necessarily the two remaining *iv*-pigs in block 1. The two necropsied C1-pigs in block two came from the same stable as the two *iv*-pigs necropsied at 56 dpi in block 1. Pigs were sedated by injecting a high dose of barbiturates via the ear vein and were subsequently bled. During necropsy the following samples were collected in the specified order: blood, urine (directly from the bladder with a sterile syringe and needle), the longissimus muscle (pork chop), the iliopsoas muscle (tenderloin), the biceps femoris muscle (pork steak), tonsil, lymph nodes (bronchial, mesenterial and hepatic), pancreas, spleen, kidney, ileum, jejunum, colon, faeces, bile (directly from the gall-bladder with a sterile syringe and needle) and liver. Part of each tissue sample was fixed immediately in 10% neutral buffered formalin for histology and the other part was stored in tubes at -70 ℃ upon return to the laboratory for RNA extraction at a later time.

3.2.4 RNA extraction

RNA was extracted in a laboratory dedicated only to RNA extraction, from 140 µl of a 10% faecal suspension, serum or urine using the QiaAmp viral RNA minikit (Qiagen, Venlo, The Netherlands). For bile samples, RNA was extracted from 100 µl using TRIzol (Invitrogen, Breda, The Netherlands). The RNA was eluted in a final volume of 35 µl elution buffer. For all tissue samples, RNA was extracted with an optimized protocol for liver samples using mechanical disruption of tissue samples with zirconium beads and subsequent silica-based RNA extraction, as described in more detail in Chapter 5.

3.2.5 RT-PCR

The reagents for the RT-PCR were prepared in a laboratory dedicated to preparing reagents for (RT-)PCR, and the RNA was added in a separate laboratory dedicated to adding RNA to the (RT-)PCR reagents. The actual RT-PCR was performed in a third laboratory that contains only thermocyclers. This segregation of laboratories, combined with the experience of the lab technician, should reduce the likelihood of RT-PCR cross-contamination.

HEV RNA in all samples was detected using a real-time RT-PCR assay targeting ORF3 [71]. This protocol was modified to analyze 5 μl of RNA by increasing the amount of 2x QuantiTect Probe RT-PCR Master Mix (Qiagen) to 12.5 μl and the amount of enzyme to 0.24 μl per reaction. For confirmation, nine real-time PCRs that generated a threshold cycle (Ct) > 38 were subjected to gel electrophoresis, showing fragments of the expected size. Non-template controls were included in each assay to monitor PCR contamination. During the experiment samples were analyzed using conventional RT-PCR directed at *open reading frame* 2 (ORF2), as described previously [128]. Decisions for transfer of pigs to other compartments and the point of autopsy at 28 days post infection for C2-pigs were based on this conventional RT-PCR. After the experiment, 588 of the 591 faecal samples were re-examined for HEV RNA with the real-time ORF3 RT-PCR used for the other samples to enable direct comparison. 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. These additional positive samples were frequently observed just before or after a series of positive samples as determined by conventional RT-PCR.

Undiluted RNA samples were examined and if samples tested HEV-negative, then undiluted RNA and 10-fold diluted RNA samples were tested to dilute possible amplification inhibitors in the RT-PCR assay.

3.2.6 Sequencing

RT-PCR fragments of the expected size were either excised from the agarose gel or were directly purified with a mini quick spin DNA column (Roche Diagnostics, Almere, The Netherlands), inserted in the pCRII-TOPO cloning vector (Invitrogen, Breda, The Netherlands), and transformed into chemically competent E. coli JM109 (Promega, Leiden, The Netherlands). After an incubation of 20±4 h at 37°C, white colonies were examined for insertion of the correct RT-PCR fragment by PCR with M13 forward and reverse primers. PCR products were analyzed by electrophoresis, for the determination of the expected size,

and were hybridized with a HEV-specific probe. Positive PCR products were purified with the QIAquick PCR purification kit (Qiagen, Venlo, The Netherlands). Sequencing was done with the BigDye Terminator Cycle Sequencing Ready Reaction (Perkin Elmer, Applied Biosystems, Foster City, Calif.).

3.2.7 ELISA

Serum samples were examined for total anti-HEV antibodies (IgTot) with a double-antigen sandwich ELISA obtained from MP Biomedicals Asia Pacific Pte Ltd. in Singapore [61]. Samples with a sample to cut-off ratio ≥ 1 were considered positive. The cut-off value equaled 0.2 plus the mean optical density (OD) of negative controls.

3.2.8 Clinical chemistry

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were analyzed longitudinally by a spectrophotometric method in an automated analyzer (HumaStar 89, Instruchemie, Delfzijl, The Netherlands) for five C2-pigs and five *iv*-pigs necropsied at 28 days postinfection, and from the two C1-pigs necropsied at the end of Block 2. The reference values in normal feeder pigs used for ALT were 15 – 46 U l^{-1} and for AST 16 – 67 U l^{-1}.

3.2.9 Histopathology

Samples of liver, gallbladder, ileum, spleen, mesenterial lymph nodes, kidney and pancreas collected from five C2-pigs and five *iv*-pigs at 28 days post infection, from the two *iv*-pigs necropsied at 56 dpi and the two C1-pigs necropsied at 55 and 65 days after first faecal HEV RNA excretion. The formalin-fixed samples were embedded in paraffin wax using routine procedures, sectioned at 4 µm and stained with hematoxylin and eosin.

3.2.10 Statistics

In total, five time-related parameters were estimated with survival analysis [81]. These include: time until first faecal HEV RNA excretion, time until first HEV-detection in serum, time until antibody development, duration of faecal HEV RNA excretion and duration of viremia. The period until first HEV-excretion for contact-pigs was estimated from days post exposure (dpe). For C1-pigs, exposure was assumed to start at the midpoint of the interval between the last HEV-negative and first HEV-positive faecal sample of the respective *iv*-pig. For C2- and C3-pigs, exposure started on the day the C1- or C2-pig, respectively, was introduced. All other time-related parameters were estimated relative to the first day of faecal HEV RNA excretion, which was assumed to start at the midpoint of the interval between the last HEV-negative and the first HEV-positive faecal sample. This relative

comparison was chosen because the moment of infection for contact-infected pigs is unknown, impeding the use of dpi. By using the onset of faecal HEV RNA excretion as a reference point the course of infection is normalized for all pigs. This normalization makes comparisons between pigs more appropriate.

To assess differences between contact-infected and inoculated pigs, data of the C2- and C3-pigs were pooled, as exposure to HEV was assumed to be similar. The C1-pigs, however, joint compartments with *iv*-pigs from inoculation onwards, displaying a different exposure-pattern than C2- and C3-pigs. Therefore, statistical differences between C1-pigs and C2/3-pigs were examined.

The differences in Kaplan-Meier (KM) survival curves for the five parameters were tested using the Log-rank statistic (α=0.05) for the three groups: C1-pigs, C2/3-pigs and *iv*-pigs. To draw conclusions on differences between contact-infected and *iv*-pigs while accounting for the variation between the two blocks, joint *p*-values were calculated from the *p*-values per parameter per block using Fischer's method [47] for computing the following statistic:

$$-2\sum_{i=1}^{2}\ln(p_i) \tag{1}$$

This statistic is chi-square distributed with degrees of freedom twice the number of *p*-values added.

Trends for increasing AST- and ALT-levels in time were assessed per pig by linear regression, with AST- or ALT-levels as continuous response variable and sampling number as continuous explanatory variable. All statistical analyses were done using SAS (version 9.1; SAS Institute, Cary, NC, USA).

3.3   Results

3.3.1   Statistics describing the course of HEV infection

The time until and the duration of faecal HEV RNA excretion and viremia, and the time until antibody development were estimated for contact-infected and *iv*-pigs (Table 2). No statistical differences were observed between C1-pigs and C2/3-pigs (data not shown), and therefore data for all contact-infected pigs were pooled. Statistical differences between the

Table 2. Differences in five parameters describing the course of HEV-infection in contact-infected and intravenously inoculated pigs

| Days...	Contact infected	Intravenously inoculated	P-values		
			Block 1	Block 2	Joined[*]
...until faecal HEV RNA excretion	7.2 (4.8 – 9.6)	3.2 (2.0 – 4.3)	0.013	0.020	0.002
...with faecal HEV RNA excretion	23.3 (18.7 – 27.9)	39.9 (27.7 – 52.1)	0.021	0.275	0.036
...until viremia[†]	12.6 (8.3 – 17.0)	3.8 (2.2 – 5.4)	0.052	0.048	0.017
...with viremia	10.5 (8.1 – 13.0)	26.2 (16.6 – 35.8)	0.089	0.345	0.137
...until antibody development[†]	13.0 (10.3 – 15.6)	12.5 (10.4 – 14.6)	0.067	0.300	0.097

[*] Fisher's joint *p*-value

[†] since first faecal HEV RNA excretion

two blocks were observed for the five parameter estimates. Statistical differences were observed between contact-infected pigs and *iv*-pigs for two parameters in both blocks, whereas one parameter differed significantly between contact-infected pigs and *iv*-pigs in one block only (Table 2). To conclude whether an overall difference between contact-infected pigs and *iv*-pigs existed, joint *p*-values were calculated. Overall differences were observed for time until onset of faecal HEV RNA excretion and viremia, and duration of faecal HEV RNA excretion.

From the first faecal HEV RNA excretion onwards, the course of the HEV RNA-titer in faeces and serum, and the development of detectable antibodies were similar for *iv*-pigs and contact-infected pigs (Figures 1 and 2).

### 3.3.2 AST and ALT levels in serum

No instant elevations in serum ALT- or AST-levels were observed for the seven contact-infected pigs and five *iv*-pigs when the values obtained were related to the baseline values for fattening pigs (data not shown). However, the presence of an increasing trend was assessed per pig by ordinary linear regression, showing a statistically significant ($p<0.05$) increase in serum ALT-levels during the period of faecal HEV RNA excretion for one contact-infected pig necropsied 55 days after first faecal HEV RNA excretion and for three *iv*-pigs (nos. 6, 7 and 9 in Tables 2 and 3). The differences in minimum and maximum values for these pigs were 14, 42, 38 and 29 U l^{-1}. The other 8 pigs did not show an increasing trend for ALT-levels and none of the pigs for AST-levels.
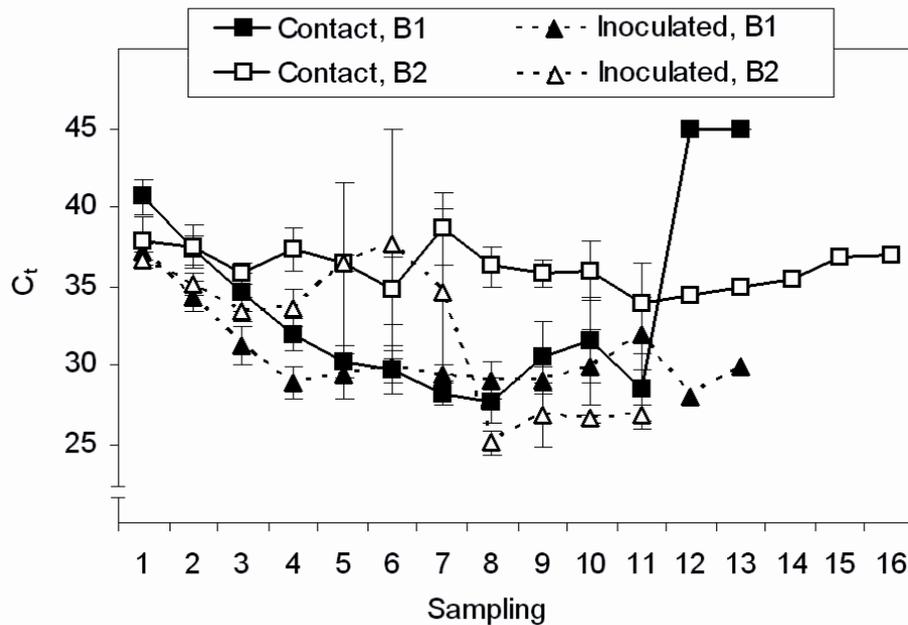
Figure 1. The pattern of faecal HEV RNA excretion in time. The pattern is represented by the threshold cycle (Ct) of the realtime RT-PCR per block (B1: Block 1, B2: Block 2) for contact-infected and *iv* inoculated pigs. The Ct values were used as relative marker for the amount of HEV in samples, under the assumption that efficiencies of the assay for all faecal samples are comparable. The first HEV-positive faecal sample is taken as starting point of the pattern (*i.e.* sampling 1) and three samplings represent 7 days. Ct values were averaged for contact-infected or *iv* inoculated pigs per sampling. Error bars indicate the standard error of the mean; absence of error bars means that only one value was available for that sampling. Note that a lower Ct usually indicates a higher HEV-concentration and Ct >40 were set at 45, because 45 cycles were completed in the RT-PCR assay.

3.3.3 HEV RNA in tissues, urine and bile

Five C2-pigs and five *iv*-pigs were necropsied at 28 days post infection to collect tissue samples, urine and bile (Table 3). HEV RNA was found less often in samples of C2-pigs (24 of 74) than in samples of *iv*-pigs (58 of 74). Remarkably however, urine of three C2-pigs tested HEV RNA-positive (with Ct values of 37, 38 and 31 for pigs no. 1, 4 and 5, respectively), as opposed to none of the *iv*-pigs. Sequences of the RT-PCR fragments were obtained for the three samples by cloning and sequencing, and were homologous to the inoculated HEV strain.

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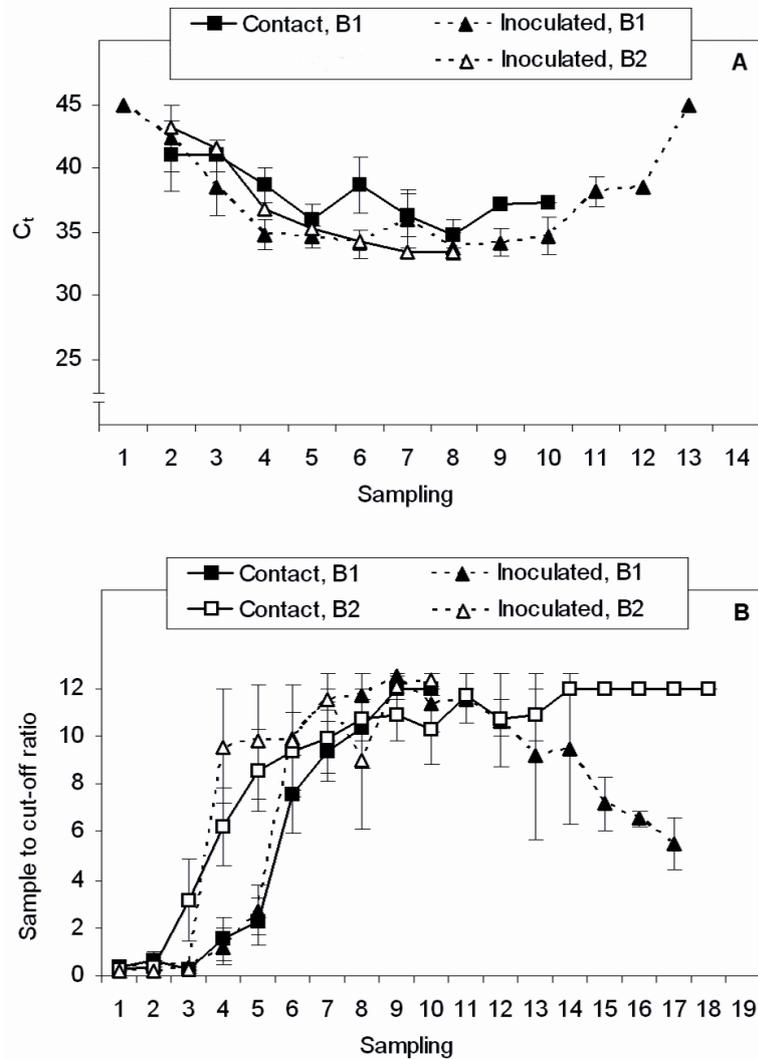
Figure 2. The pattern of HEV viremia (A) and anti-HEV antibody (total Ig) development for contact-infected pigs and iv inoculated pigs (B). The first HEV-positive faecal sample is taken as starting point of the patterns (*i.e.* sampling 1) and two samplings represent 7 days. Ct and sample to cut-off ratios were averaged for contact-infected or iv inoculated pigs per sampling. Note that a lower Ct usually indicates a higher HEV-concentration and Ct >40 were set at 45. Error bars indicate the standard error of the mean; absence of error bars indicates that only one value was available for that time-point. As only four contact-infected pigs in Block 2 showed viremia with scattered times of onset, no graph could be produced for these pigs.

Table 2. HEV RNA in organs, excreta and bile from second-generation contact-infected pigs and intravenously inoculated pigs at 28 days post infection.

Sample	Contact infected pig no.						Inoculated pig no.					
	1	2	3	4	5	Total	Total	6	7	8	9	10
Faeces	+	–	–	–	+	2/5	5/5	+	+	+	+	+
Urine	+	–	–	+	+	3/5	0/5	–	–	–	–	–
Serum	–	–	–	–	+	1/5	4/5	+	+	–	+	+
Bile	+	–	–	–	+	2/5	5/5	+	+	+	+	+
Liver	+	–	–	–	+	2/5	5/5	+	+	+	+	+
Mesenterial LN	+	–	–	–	+	2/5	5/5	+	+	+	+	+
Bronchial LN	+	–	–	–	+	2/5	5/5	+	+	+	+	+
Hepatic LN	+	–	–	+	+	3/5	5/5	+	+	+	+	+
Pancreas	–	–	–	–	–	0/5	0/5	–	–	–	–	–
Spleen	+	–	–	+	+	3/5	5/5	+	+	+	+	+
Kidney	–	–	–	–	+	1/5	4/5	+	+	–	+	+
Ileum	–	–	–	–	.	1/4	4/5	+	+	–	+	+
Jejunum	–	–	–	–	+	1/5	4/4	+	+	.	+	+
Colon	+	–	–	–	+	2/5	4/5	+	+	–	+	+
Tonsil	–	–	–	–	–	0/5	3/5	+	–	–	+	+

'+' indicates the presence of HEV RNA; '–' indicates the failure to detect HEV RNA; '.' indicates that samples were not examined

At 56 dpi, HEV RNA was detected in urine, the bronchial and hepatic lymph nodes and the spleen of both *iv*-pigs. Furthermore, HEV RNA was present in the faeces of one of the two pigs, the other showed HEV RNA in the ileum, jejunum and the mesenteric lymph node. The C1-pig necropsied at 55 days after first faecal HEV RNA excretion showed HEV RNA in the kidney and the C1-pig necropsied at 65 days showed HEV RNA only in urine. Interestingly, both urine specimens of the *iv*-pigs and one urine specimen of the C1-pig contained HEV RNA at necropsy. The urine specimen of the *iv*-pigs both showed a Ct>40, whereas the specimen of the C1-pig showed a Ct of 32.5. This C1-pig had stopped excretion of HEV in faeces about 4 weeks earlier. Clones with correct inserts were obtained for RT-PCR fragments detected in urine of the C1-pig. Subsequent sequencing showed homology to the HEV sequence of the inoculated strain.

3.3.4 HEV RNA in muscle samples

HEV RNA was detected in 12 of 20 biceps femoris muscle samples, in 11 of 20 iliopsoas muscle samples and in 9 of 20 longissimus muscle samples at various time-points after infection (Table 4). Muscle samples were found positive in contact-infected pigs up until 32 days after first faecal HEV RNA excretion. Out of 14 liver-positive pigs, 13 tested positive in at least one muscle sample (and eight in all three muscle samples), while five out of six liver-negative animals tested negative in all muscle samples. In addition, HEV RNA was detected

Table 3. HEV RNA in samples of muscle, liver and serum at various days since first faecal-HEV excretion in contact-infected pigs (C1, C2 and C3) and intravenously inoculated pigs (*iv*).

Day[*]	Type	Pig ID	Type of muscle			Liver	Serum
			Longissimus	Biceps femoris	Iliopsoas		
13	C3	21	+	+	−	+	.
13	C3	22	+	+	+	+	+
15	C3	23	+	+	+	+	+
18	C3	24	−	−	−	−	−
19	C2	19	+	+	+	+	.
23	C1	15	−	−	+	+	+
24	C1	16	+	+	+	+	.
25	*iv*	6	−	+	+	+	+
25	*iv*	7	+	+	+	+	+
25	*iv*	8	−	+	−	+	−
25	*iv*	10	+	+	+	+	+
27	C2	3	−	−	−	+	−
27	C2	5	+	+	+	+	+
27	*iv*	9	+	+	+	+	+
29	C1	17	−	−	−	−	.
30	C2	4	−	−	−	−	−
31	C1	18	−	+	−	−	.
32	C2	20	−	−	+	+	+
53	*iv*	14	−	−	−	−	−
55	*iv*	13	−	−	−	−	−

'+' indicates the presence of HEV RNA; '−' indicates the failure to detect HEV RNA; '.' indicates that samples were not examined

* since first faecal HEV RNA excretion

in examined serum samples in all but one pig (no. 8) that showed HEV RNA-positive muscle samples.

3.3.5 Histopathology

No gross pathological changes were observed in any of the examined pigs. By histopathology, moderate, multifocal, lymphohistiocytic hepatitis was observed in livers from two of five C2-pigs (numbers 4 and 5 in Table 3) and three of five *iv*-pigs (nos. 6, 9 and 10) after necropsy at 28 days postinfection. In addition, slight subepithelial lymphohistiocytic cell infiltrations were observed in the gall bladder of one C2-pig and one *iv*-pig necropsied at 28 days post infection, and of one C1-pig necropsied at 55 days after first faecal HEV RNA excretion. In the ileum a mild or moderate hyperplasia of Peyer's patches was observed in four of four examined C2-pigs, four of four examined *iv*-pigs and three of four pigs necropsied at the end of the study. A mild or moderate hyperplasia in lymph nodes was observed in one C2-pig (no. 5), two *iv*-pigs (nos. 6 and 7) and the C1-pig

necropsied 65 days after first faecal HEV RNA excretion. No lesions were observed in the spleen and pancreas.

3.4 Discussion

In the current study, HEV infection in pigs due to contact-exposure to HEV-infectious pigs was studied as model for natural transmission to describe the natural course of HEV infection in pigs. The use of intravenous HEV inoculation as initiator of the transmission process enabled collaterally the identification of differences in the course of infection due to infection route. Furthermore, HEV RNA was detected in several porcine tissues, muscles and excreta.

We present evidence of HEV RNA in urine in the current study, with 6 of 14 urine samples containing HEV RNA compared to 8 of 14 faecal samples collected at the same time. A single observation of HEV RNA in urine of a pig was reported before by Banks *et al.* [8]. Because urine samples in the current study were collected directly from the bladder using a sterile syringe with needle, contamination by HEV from other sources is unlikely. One HEV RNA-positive urine sample was obtained from a contact-infected pig at 65 days after onset of faecal HEV RNA excretion, showing a comparable Ct to that of faecal samples collected during the acute phase of infection. This finding may suggest that HEV-excretion via urine lasts longer than faecal HEV RNA excretion, and/or that urinal HEV-excretion occurs at a later stage of infection. This issue should be resolved by monitoring longitudinally the presence of HEV in urine of contact-infected pigs.

The presence of HEV RNA in urine suggests that HEV may be transmitted via urine. Until now, faecal-oral transmission of HEV is considered the main transmission route among pigs. The possibility of other transmission routes has been discussed [104, 54, 77], but efforts to experimentally transmit HEV between pigs via tonsil and nasal secretions, and HEV-contaminated needles have been unsuccessful [76]. The physical condition of urine yields easier distribution of HEV throughout a pen or stable than with faeces. In addition, the amount of urine excreted per pig per day is 5-fold the amount of excreted faeces [23]. Transmission of HEV via urine might occur orally, or aerogenically via droplet aspiration to the respiratory tract. For urine to be a transmission route, however, the HEV RNA should originate from infectious HEV particles, which is currently unknown. The detected HEV RNA may represent HEV that is bound to antibodies for disposal; anti-HEV antibodies have been observed in urine samples of human hepatitis E patients [70]. Whether or not urine contains infectious HEV could be examined by performing cell infection experiments [38, 151] or experimental inoculation of susceptible pigs with a HEV-positive urine sample.

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HEV RNA was detected in samples from organs and muscle. These samples may contain HEV RNA either extrinsically (on the surface) due to cross-contamination during necropsy or intrinsically (within the tissue). During necropsy, the organs containing excreta (faeces and bile) that can harbour high loads of HEV were sampled last to reduce the potential for extrinsic contamination. Although the contribution of these excreta cannot be excluded entirely, the most likely source for extrinsic contamination is blood. This reasoning holds especially for the muscle samples, as these were taken outside of the abdominal cavity before any other organ sample was collected. Indeed, eight of nine examined pigs with HEV-contaminated muscle samples were viremic, suggesting a potential role of blood in contamination. Whether this role involves intrinsic and/or extrinsic contamination remains to be examined, for instance by immunohistochemistry [53].

More than 50% of the muscle samples examined in the current study contained HEV RNA. Previously, HEV RNA was detected in skeletal muscle in pigs that were inoculated *iv* with HEV of genotype 3 obtained from a human patient, but not with HEV from swine [173]. The muscle samples examined in the current study were proxies for commercial pork meat (pork steak, tenderloin or pork chop). Positive samples were found until four weeks after onset of faecal HEV RNA excretion, which suggests that HEV RNA-contaminated meat could only enter stores when new HEV-infections occur later in the fattening period. For Japanese pig farms it was estimated that about 5% of the pigs were infected after five months of age [134]. In a Dutch slaughterhouse, 14% out of 80 sampled pigs excreted HEV faecally, suggesting these pigs were in the acute phase of infection (Rutjes *et al.*, unpublished data). From a public health perspective, it should be considered whether contaminated pork meat at retail indeed contains infectious HEV and what fraction remains infectious until consumption. It is advisable, however, to cook pork properly prior to consumption.

In the current study, the course of HEV infection in pigs was described for contact infected pigs. Simultaneously, these data were collected from *iv* inoculated pigs, enabling comparison between the two. The duration of faecal HEV RNA excretion was exceeded by about 16 days for *iv*-pigs compared to contact-infected pigs. In contrast, the delay in onset of faecal HEV RNA excretion was reduced by about 4 days for *iv*-pigs compared to contact-infected pigs. This reduction may, however, be partially explained by the required exposure-time for infection, which is absent in the estimate for *iv*-pigs. From the current transmission study, the rate of transmission per day was estimated at 0.66 (95% confidence interval: 0.32 – 1.35) [14]. The reciprocal of this figure gives the average exposure-time required for infection, which equals 1.5 (0.7 – 3.1) days. With the delay being 4.1 days (95% CI: 0.6 – 7.6), the exposure time explains only partially the delay. The remainder may be explained by the difference in route of infection. Other differences possibly attributable to route of infection were time until viremia and the higher number of HEV RNA positive

organs in *iv*-pigs. The observed differences suggest a more severe infection after *iv* inoculated pigs compared to contact-infected pigs. Whether these differences are important to consider in planning future experiments will depend on the aim. For instance, when using swine as a bioassay to detect infectious HEV [77] these differences will not be important, whereas in risk assessment studies the risks needs to be estimated as accurately as possible and the differences are important.

The HEV titers in serum and faeces were similar between *iv*-pigs and contact-infected pigs in the current study once faecal HEV RNA excretion had started. Meng *et al.* [105] described that HEV titers in faeces increased about 2 $\log_{10}$ (representing 6.6 Ct units under ideal real time RT-PCR conditions) when the inoculated HEV-dose increased 1,000-fold. This relationship would suggest that the HEV-doses for contact-infected pigs and *iv*-pigs in the current study were comparable and that contact-infected pigs ingested sufficient HEV to result in about 10^4 HEV RNA particles entering the bloodstream. Variation in ingested HEV-doses, however, seems likely for contact-infected pigs, because their ingestion of HEV was uncontrolled. If the doses differed, then similar HEV titres for contact-infected and *iv*-pigs suggest a plateau level of HEV released by infected cells. It has been hypothesized that HEV-release from infected cells may be caused by the immunological response rather than the cytopathic effect of HEV on hepatocytes [68]. In this perspective, the apparent plateau might reflect the maximal effect of the immune system. The current data, however, leave this issue unresolved.

Antibody development was detected in the current study at the earliest at about two days after first faecal HEV RNA excretion. The average time until antibody development was two weeks after first faecal HEV RNA excretion (contact-infected pigs) or inoculation (*iv*-pigs). Reported times until IgG development for *iv* inoculated pigs were two weeks after inoculation at the earliest [105, 54], but more frequently reported are times between three and eight weeks postinoculation [104, 77]. The difference between previous studies and the current study may be caused by the principle of the ELISAs used. In the current study, the double antigen sandwich ELISA detects IgM and IgA in addition to IgG. The IgM antibody contains 10 antigen binding sites as opposed to two for IgG, for which the likelihood to bind at least one immobilized antigen and one conjugated antigen is higher for IgM than for IgG, as discussed by Rutjes *et al.* (unpublished data). As the onset of IgM generally precedes the onset of IgG after infection, total antibody development is likely detected earlier than IgG development only. This advantage could be used in *e.g.* seroprevalence screening to minimize misclassification of the subject due to the delay in antibody development.

Histopathological lesions were observed in the liver, gall bladder, ileum and lymph nodes of HEV-infected pigs in the current study. Four pigs showed an increasing trend in AST and

ALT levels, which might suggest a slowly progressive development of liver damage during HEV-infection. Due to absence of control pigs in the current study these abnormalities cannot be attributed conclusively to the HEV-infection. However, mild hepatic lesions were previously associated with subclinical hepatitis E in naturally infected pigs [106, 27, 95] and experimentally infected pigs [104, 54, 79]. Therefore, a subclinical HEV-infection in domestic pigs may initiate energy-requiring recovery processes, possibly at the expense of production aspects such as growth rate, feed conversion or time to first estrus. Effects of HEV-infections on litter size and preterm abortion in pregnant gilts were absent [79], but effects on the above-mentioned production parameters have not been examined. This information is needed to evaluate from an economical perspective on whether intervention strategies for the reduction of the HEV-incidence among pigs can be beneficial to pig farmers.

3.5   Conclusions

The course of HEV infection in contact-infected pigs was characterized by estimation of onset and the duration of faecal HEV RNA excretion and viremia, time until antibody development, the course of AST/ALT-levels and localization of HEV in tissues and organs. HEV RNA was detected in urine of 4 of 7 contact-infected pigs and may be a source for HEV-transmission. Furthermore, 32 of 60 meat samples contained HEV RNA, suggesting the possibility of foodborne transmission to humans via pork products. Additional studies are required to assess whether urine and meat contain infectious HEV and to assess the currently unknown public health risk by contact with pigs and by consumption of pork.

3.6   Acknowledgements

Chapter 4

Estimation of the likelihood of faecal-oral HEV transmission among pigs

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ABSTRACT

The sources for human hepatitis E virus (HEV) infections acquired in developed countries are largely unknown. Pigs are potential animal reservoirs for HEV-strains that are similar to those infecting humans. Intervention at pig farms may be desired when pigs are confirmed as source for human infections, requiring knowledge about the transmission routes. Low infection rates for oral inoculation of pigs with HEV questions the efficiency of the faecal-oral route. The current study quantifies the contribution and likelihood of faeces to cause new HEV-infections in pigs due to oral ingestion. By modeling the fate of HEV in the faecal-oral pathway the daily infection risk for pigs was estimated for five scenario's that differed on a log-scale in the ingested HEV-dose or infectivity per orally ingested HEV particle (referred to as 'TMF'). Giving these parameters the values deemed most plausible based on current knowledge (TMF=1) the daily risk of infection was 0.85 (95% interval: 0.03-1). The associated expected number of new infections per day was ~4 (95% interval: 0.1 - ∞) compared to 0.7 observed in a transmission experiment with pigs, and the likelihood of faeces causing the transmission approached 1. Also in alternative scenarios the faecal-oral transmission of HEV was very likely to be the main route, with a TMF=10^{-2} leading to similar expected numbers of newly infected pigs in the experiment and the current study. The contribution and likelihood of the faecal-oral route, however, decreased by decreasing TMF, allowing for at most 94% of infections to be caused by additional transmission routes, for instance urine. Nevertheless, in all our scenarios faecal-oral transmission was expected to contribute strongly to HEV transmission. Thus, our study showed that the faecal-oral route is likely to cause HEV transmission among pigs.

4.1 Introduction

Hepatitis E virus (HEV) is the causative agent of hepatitis E among humans in developing and developed countries [122]. The virus has a single stranded, positive sense RNA genome of approximately 7.2 kilobases and can be classified into four distinct genotypes that infect humans (1 through 4) [91]. In general, infections with genotype 1 and 2 of HEV are acquired in developing countries, whereas those with genotypes 3 and 4 are acquired in developing as well as developed countries [91]. The HEV-sources for sporadic HEV infections acquired in developed countries are mostly unidentified [66, 24, 12], but person to person transmission of HEV seems to be uncommon[12]. Animal species such as wild boar, deer and pigs have been suggested as HEV source [52], possibly leading to human exposure to HEV through *e.g.* direct contact with infected animals [107, 175, 13], contact with untreated sewage of animal origin at slaughter [117] and through consumption of animal meat or organs [153, 180, 97, 15, 43, 170]. Especially domestic pigs have been studied extensively for HEV infection, and HEV is found worldwide on fattening pig farms [52]. The prevalence of HEV infection among pigs can be high, with HEV RNA being found on 55% of 97 randomly selected Dutch finishing pig farms [128] and within-herd prevalences of anti-HEV antibodies up to ~90% on Japanese pig farms [147]. Furthermore, HEV spreads between pigs (*i.e.* the basic reproduction ratio exceeded one significantly), showing the potential of pigs to be a true animal reservoir [14]. Thus, the pig-to-pig transmission of HEV may be a public health concern and intervention at pig farms could help to reduce foodborne or contact-exposure of humans to HEV.

Intervention can be applied at different stages of production, *e.g.* on pig farms or during the consumer phase. During the consumer phase, the probability of HEV infection per consumption event is likely to be decreased by proper heating [44] and by preventing cross-contamination through cutlery and cutting boards. Intervention in the consumer phase, however, will not affect risks associated with prior phases of pork production, such as professional exposure to swine. HEV-exposure during these phases may be reduced by developing and implementing intervention measures on pig farms. A reduction in the numbers of infected pigs on farms decreases the probability of having contact with an infected pig and of consuming pork products that are intrinsically contaminated by HEV. Possible strategies for reducing the HEV-incidence on pig farms include the development of a vaccine which reduces HEV-transmission sufficiently to prevent epidemics, the prevention of introduction of HEV on pig farms, or the reduction of transmission by strict hygiene protocols. However, to effectively target a reduction by these measures the sources and routes of HEV transmission among pigs need to be identified.

Infectious HEV is present in faeces from pigs and *iv* inoculation of HEV contaminated faeces leads to newly infected pigs [76]. These findings, together with the abundant presence of HEV RNA particles in pig faeces and the rapid spread of HEV among pigs due to contact-exposure [14], suggest that faecal-oral transmission of HEV genotype 3 occurs. In contrast, however, oral inoculation of faeces with HEV genotype 3 rarely leads to infection in pigs [77, 76]. These observations suggest that the faecal-oral route is not very efficient in HEV transmission and that other possible HEV-sources explain the rapid HEV-transmission among pigs. Infectious HEV can be present in blood, as pigs can be infected by *iv* inoculation of HEV-contaminated blood [76] and HEV RNA has been detected in urine [16]. Thus, in theory at least, other excreta may contribute to HEV transmission. Especially urine may be important, given the volume that is excreted and its possible HEV RNA titer [16]. Quantitative data, however, lack to conclude on the importance of the faecal-oral route in HEV-transmission.

The current study aims to assess the likelihood and contribution of faeces as source for HEV transmission among pigs using quantitative microbial risk assessment. First, the dose response relation for infection of pigs by HEV was assessed using published data. Next, the daily ingested HEV-dose due to oral ingestion of faeces was estimated for pigs and the number of newly infected pigs per unit time calculated. Finally, because modeling depends heavily on the accuracy of the input data, on the model assumptions, and on the use of the correct model structure, the estimated number of newly infected pigs with the model was verified with the number obtained from a study on HEV-transmission among pigs [14].

4.2 Methods

4.2.1 Probability of infection

A dose-response relationship was constructed from data of several experiments in which pigs were administered different HEV-doses intravenously (*iv*) (Table 1). All studies used the same methods for quantifying HEV in faeces, thus all viral doses were measured on the same scale. Pigs were considered infected when HEV RNA was detected in faeces, because it is then likely that HEV has infected cells and has been replicating. The hypergeometric and exponential dose response model were fitted to these data. The exponential model provided a fit comparable to the hypergeometric model, as judged by the likelihood ratio test. Therefore, the exponential dose-response was used to estimate the probability of infection ($P_{inf,sim}$) given a certain HEV-dose through faeces (D):

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

Table 1. Dose-response data for HEV in pigs. Number of genome equivalents (GE) inoculated intravenously in pigs and number of pigs that subsequently excrete HEV faecally. One genome equivalent represents the number of HEV genomes present in the highest serial dilution positive by RT-PCR [104].

| Dose in GE | Number of pigs | | Reference |
	inoculated	with faecal HEV excretion	
10^6	15	15	[54]
10^6	3	3	[76]
10^6	3	3	[77]
10^6	12	12	[79]
10^5	2	2	[105]
2.5×10^4	2	2	[78]
2.5×10^2	2	0	[78]
10^2	2	2	[105]
40	1	1	[104]
10	2	0	[105]
1	2	0	[105]

As the estimated value for *r* was based on *iv* inoculation, and faecal-orally transmitted HEV is at least 10^4 times less infective than *iv* inoculated HEV [77], the probability of infection per HEV particle was divided by 10^4 to adjust for oral ingestion.

4.2.2 HEV concentration in faeces

The concentration of infectious HEV per g of faeces (*C*) depends on two parameters. Firstly, faeces excreted by infectious pigs contain an amount of RT-PCR detectable units (PDU) (*N*). This *N* represents an unknown number of HEV RNA genomes (*λ*). Secondly, one PDU represent both intact and defective virus and only the fraction of intact virus (ɸ) contribute to HEV transmission. The resulting equation for *C* is then

$$C = \lambda \cdot N \cdot \varphi \tag{2}$$

where *λ* represents the number of genomes represented by one PDU.

The number of infectious HEVs per gram of faeces decays over time due to inactivation. The rate of inactivation is expressed as the average decimal reduction rate per day (δ) multiplied by the time (*t*) in days required for faeces to be ingested by a susceptible pig and affects the total number of infectious HEVs per gram of faeces as:

$$I(t) = C \cdot 10^{-\delta \times t} \tag{3}$$

where I represents the average number of infectious HEVs per gram of faeces.

The total number of ingested infectious HEVs per day subsequently depends on the amount of faeces ingested per pig per day (W), adjusted for the probability that the ingested faeces originates from the infectious pig by dividing by the number of pigs per pen (k). Thus, the daily ingested dose of HEV via faeces (D) for pigs equals:

$$D = \frac{I \cdot W}{k} \tag{4}$$

Accordingly, the following parameters needed to be quantified (Table 2):
- Number of HEV PDU excreted per gram of faeces (N)
- Fraction of HEV PDU that represents infectious HEV (ϕ)
- Number of HEV genomes per PDU (λ)
- Inactivation rate of HEV in faeces at room temperature (δ)
- Average number of pigs per pen (k)

A Monte Carlo sample of 10,000 values was drawn from the distribution of N and D was calculated for all simulated parameter values using Mathematica 6.0 (Wolfram Research, Inc., Oxfordshire, UK). The assumptions associated with the model are listed in Table 3.

Table 2. Definition of parameters and distributions used to estimate the daily infection risk for pigs with hepatitis E virus through faeces.

	Parameter	Distribution	Mean	95% range
N_f	HEV PDU per g of faeces	Gamma(0.541, 311763)	2×10^5	$300 - 8 \times 10^5$
γ	Performance of the real-time RT-PCR assay	None (constant)	50	n.a.
τ	Greater sensitivity of real time over conventional RT-PCR	None (constant)	50	n.a.
ϕ	Fraction of infectious HEV among HEV PDU	None (constant)	0.10	n.a.
k	Number of pigs per pen	None (constant)	12	n.a.
V_f	The amount of daily ingested faeces in grams	Evaluated from $0 - 100$	n.a.	n.a.
P_{inf}	Probability of infection per infectious HEV particle	None (constant)	1.3×10^{-5}	n.a.

Table 3. List of assumptions associated with the models described in this chapter.

Model parameters	
N_f	HEV particles are Poisson distributed in samples
	The efficiency of RNA extraction from faecal samples is 100%
	HEV RNA excretion by pigs in the experiment is equal to that of pigs on farms
ϕ	The fraction of infectious HEV among HEV PDU is identical between the studies providing data for the dose-response relation and the study providing data on HEV RNA excretion
	The effect of different quantification procedures among described studies on the daily probability of infection is negligible
λ	The performance of the RT-PCR can be extrapolated linearly (*i.e.* detection of one PDU represent 2500 PDU and detection of 100 PDU represents detection of 250 000 PDU)
δ_f	HEV is not inactivated during the time period considered

Dose-response relation

Each pig is equally susceptible to HEV

The probability of infection per HEV PDU after oral ingestion is 10^4 times lower than that for iv inoculation

Aggregation of virus particles does not occur or has no effect

Estimation of the number of newly infected pigs

The PDU concentration sampled initially in the Monte Carlo simulation was considered to be constant throughout the time period considered to estimate the number of newly infected pigs

Newly infected pigs do not contribute to HEV transmission (*i.e.* the number of infectious pigs per pen remains one)

HEV PDU per gram of faeces (N)

HEV PDU concentrations in faeces were estimated for faeces derived from pigs that were either contact-infected by exposure to an infectious pig or that were inoculated *iv.* (The route of infection does not influence HEV PDU concentration, as described in Chapter 3.) In total, 591 faecal samples from 36 pigs were tested with a conventional HEV RT-PCR assay as described [128] and HEV was detected in 270 samples. The most probable number approach was used to obtain maximum likelihood estimates for the HEV PDU concentration [167]. To this end, presence or absence of HEV genomes was determined in serial 10-fold dilutions of RNA samples to identify the end-point dilution (*i.e.* the dilution in which no HEV genomes could be detected anymore). Assuming HEV genomes are Poisson-distributed in samples, and accounting for the actual volume of samples tested in the conventional RT-PCR, the likelihood function for the PDU concentration was assessed. The end-point dilution was identified for 135 of the 270 HEV-positive samples, which could be used to estimate lower-

and upper limits of the HEV PDU concentration. For the other 135 HEV-positive samples, serial 10-fold dilutions were examined without obtaining the end-point dilution. These samples were generally samples with a high PDU concentration that were insufficiently diluted to reach an end-point dilution (censored data). The censored data could be used to assess the 2.5% lower limit of the HEV PDU concentration, in addition to the 135 samples with known end-point dilution. The censored data did not contain information about the upper limit of the PDU concentration. The variability in HEV concentration in pig faeces was assessed by fitting a gamma distribution to these presence-absence data. The marginal likelihood of the gamma parameters must be evaluated numerically, and maximum likelihood estimates were obtained through a grid search procedure.

Number of HEV genomes per PDU (λ)

The performance of the conventional RT-PCR that was used to assess the concentration of HEV PDUs is unknown. However, 588 of the 591 samples were re-analyzed after the experiment with a newly described real-time RT-PCR assay that reported that one PDU represents four copies of a DNA plasmid [71]. To relate this figure to the conventional RT-PCR, the difference in sensitivity between the conventional and real-time RT-PCR had to be accounted for. Therefore, a multiplication factor (τ) indicating an increased or decreased sensitivity was estimated from end-point dilutions obtained with both assays on eight RNA samples. Two additional 10-fold dilutions were detected with the real-time RT-PCR for four samples, one additional 10-fold dilution for three samples, and no additional 10-fold dilution for one sample. These findings led to an estimate of τ of 50 ($\approx 10^2 \times 4/8 + 10^1 \times 3/8 + 10^0 \times 1/8$).

The estimation of four copies of a DNA plasmid representing one PDU for the real-time RT-PCR assay does not take into account the efficiency of the enzymatic reverse transcription of RNA to cDNA. The enzymes used in the real-time RT-PCR were Sensiscript and Omniscript (Qiagen, Venlo, The Netherlands). The efficiencies of these reverse transcriptases were estimated to range between 4% in the absence of background RNA and 50% when 1 μg or 2 μg of background RNA was present [88]. These data indicate that the real time RT-PCR would require between 8 (4/0.5) and 100 (4/0.04) copies of HEV RNA as input to detect it. Faecal samples likely contain much background RNA, but also inhibitors of reverse transcription, which hampers an objective choice of either value to be used. Therefore, it was assumed that the real-time RT-PCR requires on average ~50 HEV PDUs before it is detected.

The efficiency of the reverse trancriptase used in conventional RT-PCR is unknown. If this was known, then the difference in reverse-transcriptase-efficiencies between the enzymes used in conventional RT-PCR and in real-time RT-PCR had to be accounted for. Currently,

the total number of PDU represented by one PDU detected by conventional RT-PCR was estimated as 50·τ.

Inactivation rate of HEV in faeces (δ)

No data have been reported on inactivation of HEV in faeces at room temperature (about 20°C). Data on inactivation of poliovirus in artificial surface and groundwater at temperatures of 22°C are reported by De Roda Husman *et al.* [32] The poliovirus characteristics are: a ~28 nm particle with a small, round, single stranded, positive sense RNA genome of 7.5 kilobases which is protected by a protein capsid. Decay rates for poliovirus were between 0.016 and 0.051 per day. As HEV is environmentally transmitted, like poliovirus, we assume it is similarly persistent. Furthermore, considering the rapid transmission of HEV among pigs [14] and therefore likely a short time interval between HEV-excretion and subsequent intake, it is plausible that inactivation of HEV particles has a small effect on the daily ingested HEV dose. Therefore, inactivation was not further considered in the exposure assessment.

Fraction of infectious HEV particles among HEV PDUs (φ)

The fraction of infectious HEV particles among HEV PDU can not be studied due to the absence of a cell culture system for HEV. This also means that studies describing HEV-detection rely on molecular techniques and that results are presented in PDUs and not infectious HEV particles. These studies include those that were used to construct the dose-response model in the current study. Thus, by basing the dose-response model on estimated PDUs, the translation from PDU to infectious HEV particles is implicitly present in the estimate of infectivity (*r*).

Average number of pigs per pen (k)

Any faeces excreted by non-infected pigs in a pen will be ingested with the same probability of intake as the faeces excreted by a HEV-infected pig. Thus, in effect, the HEV-contaminated faeces will be diluted *k*-fold, *k* being the number of pigs per pen. On Dutch farms, the average number of pigs per pen varies between 7 and 17, with a majority of farms housing 11 to 13 pigs per pen. The scenario of 12 pigs per pen is evaluated as baseline scenario in the current study.

4.2.3 Number of newly infected pigs by ingestion of faeces

The current study aims to quantify the probability of HEV transmission when one infectious fattening pig is introduced into a pen of susceptible pigs. The probability of infection by

ingestion of faeces per day was calculated from equation (1) with D from equation (4). It was assumed that the ingested amount of faeces was a daily intake and that virus inactivation had not occurred in the interval between excretion and subsequent intake. In an experimental transmission study [14], the probability of a new HEV-infection occurring ($P_{inf,exp}$) was estimated from the observed transmission using an SI-model, with S representing the number of susceptible pigs and I the number of infectious pigs ($S+I=N$) according to the equation:

$$P_{inf,exp} = 1 - e^{-\beta_{exp} \cdot \frac{S \cdot I}{N} \cdot \Delta t}$$
(5)

In Eqn. (5), the rate of transmission β_{exp} represents the number of new infections estimated to be caused by one infectious pig per unit time in the transmission experiment. To estimate the expected number of pigs infected per time period in the current simulation study (β_{sim}), β from Eqn. (5) was solved by using $P_{inf,sim}$ from Eqn. (1). In the simulation model, the time period is one day ($\Delta t=1$), and the probability of infection is estimated for a single susceptible pig ingesting faeces contaminated by HEV from a single infectious pig, and thus $S=I=1$. In that particular case, equation (5) can be modified into:

$$P_{inf,sim} = 1 - e^{-\frac{1}{2} \cdot \beta_{sim}}$$
(6)

Combining equations (1) and (6) shows that $-\frac{1}{2}\beta_{sim}$ equals $-r \cdot D$ and β_{sim} is calculated by $2 \cdot r \cdot D$. The β_{sim} was calculated for the mean HEV-dose and its 95% interval limits. The expected number and 95% interval of newly infected pigs were subsequently calculated per day ($t=1$) and for the entire period of faecal HEV excretion, estimated to be around 23 days at average [16]. This calculated number of newly infected pigs was compared to the number of newly infected pigs estimated from the experimentally observed β (β_{exp}) [14]. The 95% interval for the latter was constructed from the 2.5% and 97.5% percentiles of a Poisson-distribution with mean $\beta \cdot \Delta t$, representing the expected variation in number of new HEV-infections. The variation in simulated and observed numbers of newly infected pigs is compared

4.2.4 Likelihood of faecal-oral transmission

The likelihood of faeces as the cause of the 15 experimentally observed newly infected pigs after 23 days was assessed by determining the cumulative probability for the mean simulated number of newly infected pigs at 23 days given a Poisson-distribution with $\mu = 15$. The 95% interval was obtained by assessing the cumulative probability for the 95% interval of the simulated number of newly infected pigs.

4.2.5 Total multiplication factor

The influence of the individual parameter values on the results was investigated by means of Monte Carlo simulation. Changes in $P_{inf,sim}$ are subject only to changes in $r \cdot D$, and $r \cdot D$ is related linearly (multiplicative model on a log-scale) to the model parameters. Therefore, a change in $r \cdot D$ and thus $P_{inf,sim}$ by a certain factor is independent of which parameter actually changed. Therefore, the sensitivity analysis is presented for total multiplication factors (TMFs) as compared to the baseline situation (TMF=1, parameter values as described in Table 2). For instance, if the probability of infection would be 100-fold lower than assumed for the baseline situation and the ingestion of faeces would be 10 g in stead of 1 g, then the total multiplication factor would be 0.1.

The most likely TMF for the experimental observations was simulated (10^6 iterations) by representing β_{exp} as a Poisson-distribution with a mean of 15 divided by the length of the infectious period of 23 days and β_{sim} as derived above. The likelihood of the TMF is calculated as the relative frequency of the simulated ratio of β_{exp} and β_{sim}.

4.3 Results

The dose response relation for *iv* inoculation is shown in Figure 1. The probability of infection per infectious HEV particle (r in Eqn. 1) was estimated to be 4.8×10^{-3} for *iv*
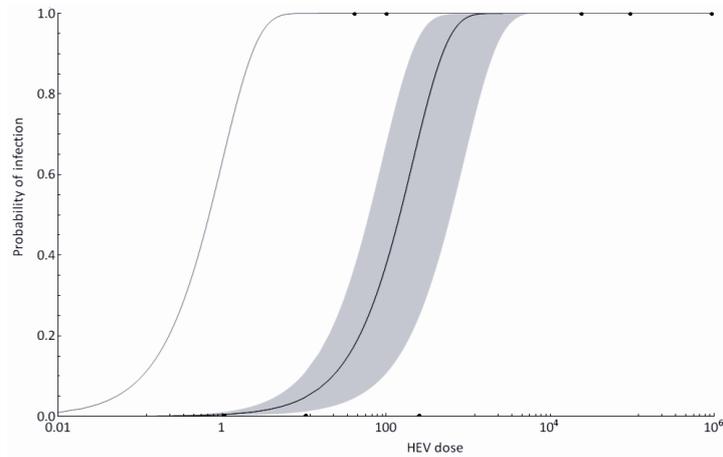

Figure 1. The dose response relationship for intravenous inoculation of HEV in pigs. The black solid line represents the mean probability, the gray-shaded area the corresponding 95% confidence interval. The thin solid line represents the maximum probability of infection (*i.e.* when the probability of infection per HEV particle would be 1) and the dots represent the observed data.
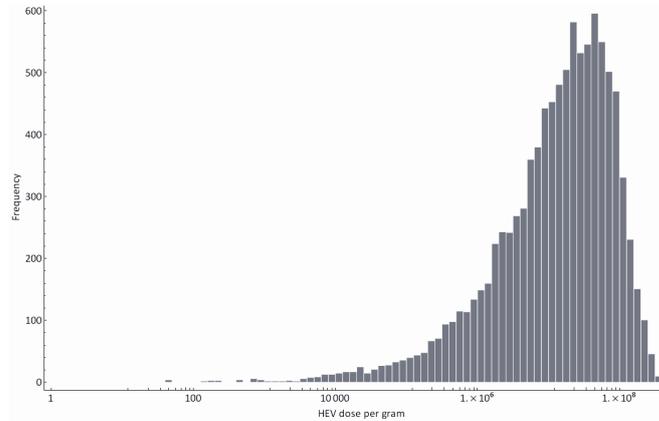
Figure 2. The distribution of number of HEV PDU per gram of faeces.

administration. The probability of infection adjusted for oral ingestion of an infectious HEV particle was 4.8×10^{-7}. The estimated orally ingested HEV-dose at which the probability of infection equals 50% is 1.4×10^6 HEV genomes.

Figure 2 shows the distribution of the HEV dose per gram of faeces when one of twelve pigs in a pen excretes HEV faecally. The mean dose per gram of faeces was estimated to be $\sim 10^8$ (95% interval: $\sim 10^6$ – $\sim 10^9$) HEVs per gram of faeces. To ingest 1.4×10^6 HEVs ($P_{inf,sim}$=0.5), about 20 mg of faeces needs to be ingested. The risk of HEV-infection due to oral ingestion
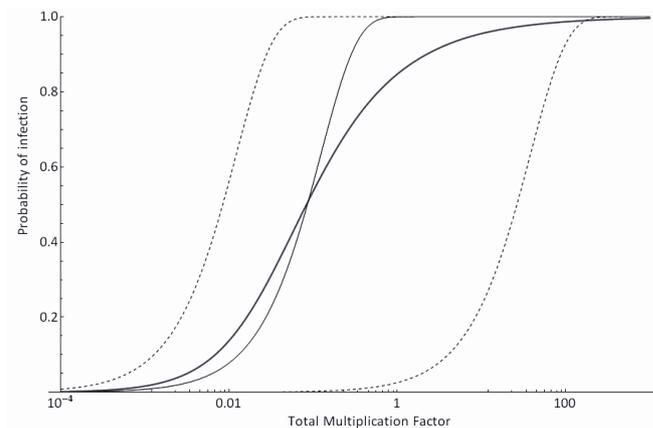
Figure 3. The mean (thick solid line), median (thin solid line) and 95% interval (thin dotted lines) probability of infection after oral ingestion of HEV through faeces as function of the total multiplication factor. In the baseline scenario (TMF=1), the probability of ingestion for 1 gram of faeces is estimated.

Table 4. Likelihood of faeces as source for HEV transmission for the current model study as estimated from an experimental study, and influence of changes in the model parameters on the estimated number of infected pigs.

| | Number of newly infected pigs after | | | | Likelihood of faeces as source for infection | |
| | 1 day | | 23 days | | | |
	Mean	95% interval	Mean	95% interval	Mean	95% interval
Model TMF						
10	6.2	$0.5 - \infty$	143	$12 - \infty$	1	$0.26 - 1$
1	3.8	$0.1 - \infty$	87	$1.2 - \infty$	1	$4.2 \times 10^{-6} - 1$
10^{-1}	1.5	$0.0 - \infty$	35	$0.1 - \infty$	1	$<10^{-6} - 1$
10^{-2}	0.3	$0.0 - 1.7$	7	$0.0 - 38$	0.55	$<10^{-6} - 1$
10^{-3}	0.03	$0.0 - 0.2$	0.8	$0.0 - 3.8$	3.5×10^{-5}	$<10^{-6} - 2 \times 10^{-4}$
*Experiment**	0.7	$0 - 3$	15	$8 - 23$		

* as reported in Chapter 2.

of 1 g of faeces per day was 0.85 (95% interval: 0.03 – 1), with 85% of the observations having $P_{inf,sim}$>0.5 (Figure 3). For the ingestion of 10 g of faeces per day with the remaining parameters fixed (TMF=10), the average estimated risk was 0.97 (95% interval: 0.55–1) with >95% of the observations having $P_{inf,sim}$>0.5.

The calculated expected number of newly infected pigs increased with on average about 4 pigs per day in the baseline scenario (TMF=1), theoretically reaching 87 newly infected pigs at the end of the infectious period of 23 days, with the variability ranging from 1.2 to infinity (Table 4).

The sensitivity analysis for the parameter values indicated that the average expected number of newly infected pigs ranged from 0.8 to 199 at the end of the infectious period of 23 days (Table 4).When the TMF decreased 100-fold, the mean estimates of the current model approached the observed number of newly infected pigs in the experiment, although the predictive intervals were larger for the model estimates compared to the estimates based on the experiment. The most likely TMF based on the experimental observations was 2×10^{-2} (95% interval: 3.5×10^{-3} – 11.8) (Fig 4). In the scenarios with a TMF of 10, 1 and 10^{-1}, the likelihood that faeces caused the new infections was on average 1, with large 95% intervals for the scenarios with a TMF of 1 and 10^{-1} (Table 4).

4.4 Discussion

In the current study, reported quantitative data on HEV infection in pigs were combined to estimate the probability of infection through ingestion of faeces by a HEV-susceptible pig.
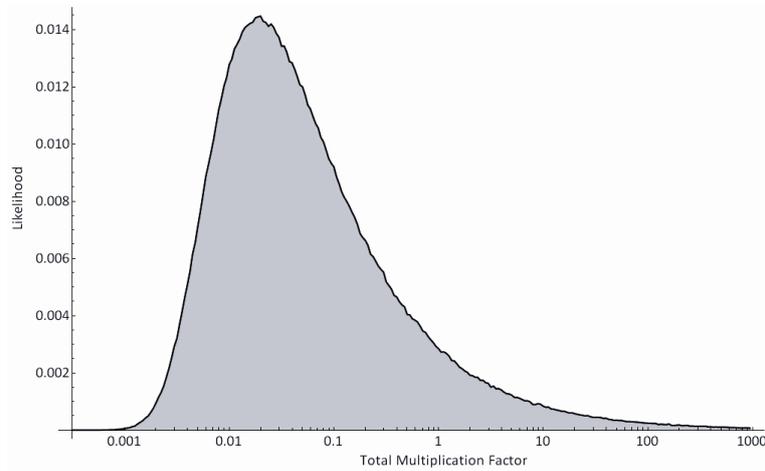
Figure 4. Likelihood of the total multiplication factor given the observations of Bouwknegt *et al.* [14] during a transmission experiments with pigs.

The dose response relation for *iv* inoculation of HEV was constructed, the ingested HEV-dose through faeces was estimated and the dose response relation was used to examine whether this dose was sufficient to cause new infections among pigs. For comparison, the estimated numbers of newly infected pigs by the model were compared to the numbers observed in a transmission study. The model supports attribution of all of the experimentally observed HEV transmission to faecal-oral transmission in the baseline scenario. Faecal-oral transmission can thus be assumed to be the main transmission route for HEV under these circumstances, even though the success rate of oral inoculation of pigs with HEV is low [77, 76].

The currently presented dose-response relationship is the first to be reported for HEV. The adjustment made to represent the dose-response after oral ingestion of HEV is based on the observation that *iv* inoculation of a certain dose of HEV genotype 3 resulted in HEV-infection in pigs, whereas oral inoculation with a 10^4 higher HEV-dose did not [77]. An equal observation was made for cynomolgus monkeys that were administered HEV genotype 1 [156]. Hence, oral ingestion may require at least a 10^4 times higher HEV-dose compared to *iv* inoculation. The only reported successful oral inoculation of a pig occurred after ingestion of about 10^8 genome equivalents (GE; for a definition see the caption of Table 1) of HEV per day at three consecutive days. In the same trial, two other pigs that received the same dose remained uninfected. The maximum likelihood estimate for the probability of infection in that case is 1/3 and this probability of infection for *iv* inoculation corresponds to an administered dose of 1.6 $\log_{10}$ HEV GE. Assuming the efficiency of infection after *iv* inoculation and oral ingestion is similar, then the estimated difference in infectivity would

be $10^8/10^{1.6} = 2.5 \times 10^6$. The difference between this estimate and the assumed factor of 10^4 in the current study is represented in the sensitivity analysis by a TMF of 10^{-2}. This TMF is close to the most likely TMF of 2×10^{-2} based on the observations from the transmission experiment.

Given the baseline scenario (TMF=1), transmission of HEV through the faecal-oral route is very likely. The variation in the simulated number of newly infected pigs after 23 days, however, shows a 2.5% lower limit of 1.2. This finding indicates that the faecal-oral route may account for only 8% (1.2/15) of the observed transmission. Part of the observed transmission must then be caused by sources other than faeces. In general, sources for transmission of an infectious pathogen can be aerosols, saliva, nasal secretions, blood, semen, urine, and consumed meat and organs. During the experiment, exchange of blood or semen, and eating meat and organs was unlikely. To date, none of the remaining theoretical sources for HEV transmission have been examined satisfactorily. Nasal and tonsil swabs have been found positive for HEV RNA [104], but a subsequent experiment could not conclude on a potential role for this transmission route [76]. Urine of contact-infected pigs can contain HEV RNA, but the infectivity of HEV in urine is unknown [8, 16]. Findings on aerosols and HEV have not been reported to date. Hence, the potential HEV-sources that may cause transmission in addition to faeces require further investigation. However, we hypothesize that given the daily excreted volume and the potential high HEV concentration, urine is most likely to serve as HEV source additional to faeces. The existence and contribution of infection routes other than faeces need to be identified to establish proper control measures to reduce HEV transmission on pig farms.

A daily intake of faeces by piglets was reported to be approximately 8 grams and 22 grams when housed on slatted and solid floors, respectively, but these intakes likely occured during nursing on faecally contaminated nipples [133, 51]. A daily faeces intake of 40 g per kg of diet was assumed to be reasonable for fattening pigs under natural conditions in another study, although this assumption was not experimentally confirmed [80]. Nonetheless, if fattening pigs consume about 2 kg of diet in the onset of the fattening phase, then the daily intake of faeces would be about 80 g. In that case, and considering the previously described adjustment of the infectivity by 10^{-6} as opposed to 10^{-4}, a TMF close to 1 would be plausible. Faeces is then very likely to explain HEV transmission among pigs.

The variability in the estimated number of newly infected pigs was large for the simulated study compared to that estimated from the experimental study. The variability in the simulated estimates is caused solely by the observed distribution of HEV PDU concentration, because variability of all other parameters was neglected. In contrast, the

variability in the expected number of newly infected pigs from the experimental study was Poisson distributed with mean $\beta \cdot \Delta t$. In the analysis of the transmission experiment the probability of infection was assumed constant, *i.e.* there is no heterogeneity among pigs. Such heterogeneity, however, is likely to exist, for instance due to variation in host susceptibility or in HEV PDU concentration per gram of faeces. This heterogeneity causes variability in the probability of infection and in the expected numbers of newly infected pigs. Therefore, the larger 95% intervals based on the risk model simulation compared to the observed transmission rates are to be expected.

In conclusion, our model simulation of the daily ingested HEV dose per gram of faeces and the subsequent translation to a risk of infection showed that the faecal-oral route is likely to cause HEV transmission among pigs. It remains unclear, however, whether faecal-oral transmission can account for all observed transmissions among pigs. To examine this aspect, more data are needed, predominantly on the probability of infection per orally ingested HEV particle and on the average ingested amount of faeces per pig per day.

4.5 Acknowledgements

Chapter 5

Hepatitis E virus RNA in commercial porcine livers in The Netherlands

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ABSTRACT

Human hepatitis E virus (HEV) infections by genotype 3 strains in industrialized countries are hypothesized to be caused by pigs. To examine this hypothesis, the potential health risks of transmission routes should be examined. Possible foodborne transmission was studied by quantifying the presence and infectivity of HEV in commercial porcine livers in The Netherlands. A comparison of four tissue disruption and seven RNA extraction methods revealed that mechanical disruption followed by silica-based RNA extraction gave the highest RNA yields and was therefore employed on commercial porcine livers. Four (6.5%) of 62 porcine livers were HEV RNA positive by reverse transcriptase PCR and Southern blot hybridization. Each positive liver was estimated to contain ~65 PCR-detectable units per g. Sequences were obtained for three of four positive livers and classified as HEV genotype 3. Ninety-three percent similarity to Dutch human HEV sequences and 97% similarity to Dutch swine HEV sequences were observed. To determine whether positive livers contained infectious HEV particles, extracts from livers with known HEV RNA sequences were inoculated intravenously in pigs. Two control pigs were included: one was inoculated with a high dose known to result in infection (104 PCR-detectable units of HEV RNA), and the other was inoculated with a lower concentration of virus that equaled the concentration of PCR-detectable units in commercial livers (~20 PCR-detectable units). Infection was observed in the high-dose control, but not in other pigs, suggesting a dose-dependent response in pigs. Hence, the implications of HEV RNA in commercial porcine livers in The Netherlands are unknown. However, HEV RNA is present in commercial porcine livers, and sufficient heating of porcine livers before consumption as precautionary measure is recommended.

5.1 Introduction

Hepatitis E virus (HEV) is an enterically transmitted RNA virus from the family Hepeviridae and causes liver inflammation in humans [114]. HEV is currently classified into 4 genotypes (consecutively named 1 to 4) and 24 subtypes. The different genotypes have different geographical distributions [91]. Genotype 1 and 2 strains are found in developing countries as a major cause of outbreaks and sporadic cases of hepatitis E [39]. In Western countries, these strains are exclusively found in patients who have visited a developing country. Genotype 3 and 4 strains are found predominantly in Western countries as causes of sporadic cases of locally acquired hepatitis E, which are increasingly observed in industrialized countries [137, 171, 18, 66, 119]. The origin of HEV in these locally acquired cases is mostly unknown, but a role for swine has been suggested on the basis of the high prevalence of HEV among pigs and the high similarity between porcine and human HEV sequences from the same geographical region[106, 171]. At present, definite evidence for zoonotic transmission from domestic pigs to humans has not been reported. However, pigs were shown to be susceptible to human HEV strains, and primates were susceptible to porcine HEV strains [105]. Furthermore, higher HEV seroprevalence estimates for groups of veterinarians and swine farm workers than for control groups were observed; thus, infection seems related to direct contact with pigs [36, 107, 175]. To examine whether swine are a source for human hepatitis E, possible transmission routes need to be examined, and associated risks need to be quantified.

Cases of foodborne transmission from wild boar and deer to humans have been reported for genotype 3 strains in Japan [153, 89]. Hence, genotype 3 strains of HEV have the potential to be transmitted via foods. The public health implication of foodborne transmission, however, is unknown, and the extent will depend on the prevalence and infectivity of HEV in food samples, the preparation of food, and the amount consumed. To evaluate if appropriate measures are required for public health protection, a microbiological risk assessment can be conducted [67]. Results from this assessment can subsequently be used to direct the development of intervention strategies aimed at reducing the morbidity or mortality due to HEV. In risk assessments, quantitative data are used preferably.

Molecular detection of viral RNA does not discriminate RNA of infectious viral particles from RNA of defective viral particles. In research focused on infectious diseases, identification of infectious particles is necessary to estimate the implications of detected viral RNA for public health. Infectivity can be determined by cell culture, which has recently been described for HEV [151]. For this system, however, a minimum viral load of HEV of 6×10^4 copies is recommended for inoculation onto cells and hence cannot be used to determine the infectivity of samples that are contaminated at low levels. Food and

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environmental samples, however, are often contaminated at low levels. An alternative approach for estimating the infectivity of molecularly detected HEV is to inoculate susceptible animals with extracts from positive samples [118, 78]. Animals susceptible to HEV infection are, among others, nonhuman primates and swine [121].

In The Netherlands, HEV RNA of genotype 3 was detected in 55% of pooled fecal samples from finishing pig farms [128]. Fecal excretion of HEV indicates that pigs are in the acute phase of infection. If pigs are close to slaughter and experience an acute phase of infection, pork products that enter stores may be contaminated by HEV. This raised concerns about the possible foodborne transmission of HEV in The Netherlands. The objective of this study was therefore to quantify the presence of HEV in commercial porcine livers in The Netherlands and to assess whether positive livers contained the infectious virus. To examine the presence of HEV in porcine livers as accurately as possible, four procedures for tissue disruption and seven procedures for RNA extraction were compared on RNA yield. The most optimal combination of disruption and RNA extraction was subsequently employed on commercial porcine livers. Detected HEV RNA was sequenced and compared with published swine HEV sequences and HEV sequences obtained from hepatitis E patients who acquired their HEV infections in The Netherlands. Infectivity of detected viral particles was examined by the intravenous inoculation of extracts from positive livers in pigs. Data from the present study can contribute to a microbiological risk assessment to estimate a possible risk of foodborne transmission of HEV.

5.2   Materials and Methods

5.2.1   Livers

Sixty-two porcine livers were obtained from Dutch butcher shops (n=56) and retail stores (n =6) from May to July 2005. A positive control liver was obtained from a pig that was inoculated intravenously with a recently isolated and characterized Dutch pig strain of HEV genotype 3 (GenBank accession number DQ996399) and that was sacrificed 17 days postinfection (dpi). The liver contained 3×10^8 PCR-detectable units (PDU) per g of liver [Bouwknegt et al., unpublished data]. The negative control was a porcine liver acquired from a local butcher shop that repeatedly tested negative for HEV RNA by reverse transcriptase (RT) PCR.

5.2.2   Tissue disruption

Three parts of inner liver were excised and homogenized manually with sterile surgical blades. Liver tissues were further disrupted mechanically with sterile beads 1 mm (Biospec Products, Bartlesville, Okla.) or 2 mm (Bio101 Inc., Carlsbad, Calif.) in diameter in 2-ml

reaction tubes. A total of 50, 150, or 250 mg of manually homogenized liver tissue and 1 ml of lysis buffer were added to a tube with beads, and these tubes were vibrated twice at 4 m/s for 40 s in a Hybaid Ribolyser Cell Disrupter (Hybaid, Ltd., Ashford, UK). Subsequently, the tubes were placed in a rack at room temperature for 10 min to settle, or proteinase K (Qiagen, Venlo, The Netherlands) was added to a final concentration of 0.35 μg/ml for additional enzymatic disruption and then incubated at 55°C for 10 min. Supernatant was transferred to a clean reaction tube for RNA extraction.

5.2.3 RNA extraction procedures

Seven procedures were selected for RNA extraction: an in-house procedure with size-fractionated silica beads (Sigma, Zwijndrecht, The Netherlands), the RNeasy Mini kit (Qiagen), the RNeasy Midi kit (Qiagen), NucliSens isolation (bioMérieux, Boxtel, The Netherlands), NucliSens magnetic extraction (bioMérieux), TRIzol (Invitrogen, Breda, The Netherlands), and a combination of phase separation with TRIzol and RNA extraction from the aqueous phase with the in-house procedure. For the in-house procedure, wash steps and elution of RNA were done as described by Boom et al. [11]. Other procedures were done as described by manufacturers, with use of the animal tissue protocol for the RNEasy Mini and Midi kits. All procedures used guanidinium isothiocyanate as a lysing component in the lysis buffer. For the Midi kit, elution of RNA from the silica membrane was done with 150 μl of elution buffer as described by the manufacturer. For all other procedures, RNA was eluted with 35 μl of elution buffer. To compare RNA yield directly, RNA in a volume of 150 μl was precipitated with ethanol and subsequently dissolved in 35 μl of elution buffer.

5.2.4 HEV RT-PCR and hybridization

For the detection of HEV RNA, a single round RT-PCR with primers ORF2-s1 and ORF2-a1 was used [161]. This RT-PCR yielded a fragment of 197 nucleotides. The RT-PCR fragment was separated in a 2% agarose gel, and suspected HEV RT-PCR fragments were confirmed by Southern blot hybridization as described [161]. Five- or 10-fold serial dilutions were prepared from undiluted RNA and included in the RT-PCR to obtain endpoint dilutions. Blanks were included after each dilution series to monitor contamination, and an internal control RNA was added during reverse transcription to monitor inhibition during RT-PCR [128]. Three serial dilutions of porcine HEV RNA of genotype 3 (GenBank accession no. DQ996399) were used as positive RT-PCR controls, with the second serial dilution containing an RNA concentration around the detection limit of the RT-PCR assay.

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5.2.5 Sequencing

Because of the presence of internal control RNA, direct sequencing of the RNA was not possible. Therefore, positive RT-PCR fragments were excised from the agarose gel, purified with a QIAquick gel extraction kit (Qiagen), inserted in the pCRII-TOPO cloning vector (Invitrogen), and transformed into chemically competent E. coli JM109 (Promega, Leiden, The Netherlands). After an incubation of 20±4 h at 37ºC, white colonies were examined for insertion of the correct RT-PCR fragment by PCR with M13 forward and reverse primers. Fragments that were of expected size and hybridized with the HEV-specific probe were purified from agarose gels with the QIAquick gel extraction kit (Qiagen). Sequencing was done with the BigDye Terminator Cycle Sequencing Ready Reaction (Perkin Elmer, Applied Biosystems, Foster City, Calif.). Phylogenetic analyses were done by BioNumerics (version 4.5, Applied Maths, Sint-Martens-Latem, Belgium). First, pairwise similarities were calculated with clustering based on UPGMA (unweighted pair group method with arithmetic mean), which was followed by the calculation of multiple alignment and assessment of global clustering by UPGMA and the Jukes-Cantor correction.

5.2.6 Experimental infection

Five domestic pigs (*Sus scrofa domestica*) that were 7 to 8 weeks old were inoculated with suspensions of livers that contained HEV RNA. Pigs were housed individually in a BSL-2 (biosafety level 2) facility and given feed ad libitum. About 2 weeks prior to inoculation, all five pigs tested HEV negative by RT-PCR on fecal samples and by double-antigen sandwich enzyme-linked immunosorbent assay (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd., Beijing, China) on serum samples.

Three pigs were inoculated intravenously with 3 to 4.5 ml of an extract from commercial porcine livers that contained HEV RNA. Two pigs were inoculated either with 3 ml of a low-dose control or with 2 ml of a high-dose control. The control virus was obtained from the liver that was used as a positive extraction control in the present study (GenBank accession no. DQ996399). To prepare extracts from livers for inoculation, eight replicates of 360 mg of liver tissue were disrupted in 800 μl of Eagle minimal essential medium (GIBCO/Invitrogen). Supernatants of the replicates were pooled, centrifuged for 15 min at 10,000 × g, and filtered through a microfilter with a 0.45-μm pore size. The low-dose control contained a viral count equivalent to the inocula made from commercial livers, as estimated from the endpoint dilution in RT-PCR.

This animal experiment was approved by the Ethics Committee on Animal Experiments of the Animal Sciences Group of Wageningen University Research in Lelystad.

5.2.7 Statistical analyses

The prevalence estimate and 95% confidence interval (CI) were calculated with exact statistics by Stata (version 9, StataCorp LP, College Station, Tex.). Estimates of RNA yield were obtained with Mathemathica (version 5.1, Wolfram Research, Inc., Champaign, Ill.) as described [159]. In short, maximum likelihood estimates of PDU per RNA extraction as most probable number were obtained from the most diluted HEV positive sample that was confirmed by hybridization of RT-PCR products. It was assumed that negative samples did not contain HEV RNA, that the dispersion of PDU in RNA samples followed a Poisson distribution, and that the detection limit of the RT-PCR was 1 PDU. Estimates of PDU in the most diluted positive sample were subsequently calculated backward following the experimental scheme to obtain an estimate of the PDU for undiluted samples.

5.3 Results

5.3.1 Comparison of tissue disruption procedures

The most efficient disruption method was identified by comparing RNA yield after disruption of liver tissue with beads of 1 and 2 mm in diameter. Furthermore, the effect of additional enzymatic disruption with proteinase K was examined. To this end, 150 mg of liver was disrupted, and RNA was subsequently extracted with the in-house procedure. Similar RNA yields of about 10^6 PDU per extraction were obtained with both types of beads in the absence of proteinase K. Addition of proteinase K increased variation between duplicates without increasing RNA yield. Therefore, subsequent disruptions of liver were done without proteinase K and with beads of 1 mm.

5.3.2 Comparison of RNA extraction procedures

Extractions of RNA based on an in-house procedure with size-fractionated silica beads, the RNeasy Mini kit, the RNeasy Midi kit, NucliSens isolation, NucliSens magnetic extraction, TRIzol, and a combination of TRIzol and the in-house procedure were compared with 150 mg of liver to identify the most efficient extraction method. Because the Mini kit has a maximum loading capacity of 30 mg, this procedure was not included in this comparison. The highest RNA yield was observed for the in-house procedure and for TRIzol (Table 1). For TRIzol, however, more nonspecific RTPCR products were observed in undiluted samples; therefore, this procedure was not examined further. Furthermore, RNA yields for different amounts of liver were compared for the in-house procedure (50 and 250 mg), the Mini kit (50 mg), and the Midi kit (250 mg). A higher input generated higher RNA yields for the in-house procedure and the Midi kit, as determined by the endpoint dilution of fivefold

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Table 1. Estimated RNA yield in PCR detectable units per 35 µl elution buffer for different RNA extraction procedures, with various amount of porcine liver as input.

Method Description	Input of liver tissue		
	50 mg	150 mg	250 mg
In house silica	$1.1×10^5$	$1.7×10^7$	$7.9×10^7$
RNeasy® Mini kit	$3.2×10^5$	nd*	nd
RNeasy® Midi kit‡	nd	$1.7×10^6$	$1.4×10^7$
NucliSens® Isolation	nd	$1.7×10^6$	nd
NucliSens® Magnetic Extraction	nd	$1.7×10^6$	nd
Trizol™	nd	$1.7×10^7$	nd
Trizol™/in-house silica	nd	$1.6×10^5$	nd

* nd: not determined; ‡ Procedure included a second elution step, followed by ethanol precipitation

serial dilutions, without an increase of inhibition of RT-PCR. The RNA yield of the in-house method was higher than the yield of the Midi kit. The Mini kit gave a higher RNA yield than the in-house procedure for 50 mg of liver but a lower RNA yield than the in-house procedure and Midi kit for 250 mg of liver. For the Midi kit, a second elution of RNA from silica was done in each extraction. Therefore, the effect of a second elution of RNA was examined for the Mini kit and in-house procedure. Because RNA was dissolved in an increased volume, it was precipitated and dissolved in 35 µl of elution buffer to enable direct comparison. A second elution decreased RNA yield for the in-house method with 0.7 ^{10}log PDU for an input of 150 and 250 mg of liver and, for the Mini kit, with 0.4 ^{10}log PDU for an input of 50 mg of liver. In conclusion, the most efficient procedure for RNA extraction was the in-house method with 250 mg of liver and a single RNA elution step. The Midi kit gave the second highest RNA yields for 250 mg of liver and the highest RNA yield of all examined commercial methods.

5.3.3 Commercial livers

The most efficient procedures of tissue disruption and RNA extraction were employed on 62 commercial livers. HEV RNA was detected in four livers with RT-PCR and Southern blot hybridization of RT-PCR products, giving a prevalence estimate of 6.5% (95% CI: 1.8 to 15.7%). HEV RNA was detected only in the undiluted samples, yielding an estimated viral load of about 65 PDU/g (95% CI: 3 to 580 PDU/g). Sequences from three of four RT-PCR products were obtained and classified as genotype 3 (Fig. 1A). Two of three sequences showed the highest similarity to Dutch swine sequences (94% for NLSWLiver21 with NLSW105 and 97% for NLSWLiver25 with NLSW28), and the third showed the highest similarity to a United Kingdom swine sequence (92% for NLSWLiver27 with P354). Within genotype 3, the existence of at least 10 different subtypes (3a through 3j) is proposed [91]. Each of the HEV sequences from Dutch commercial porcine livers belonged to a different
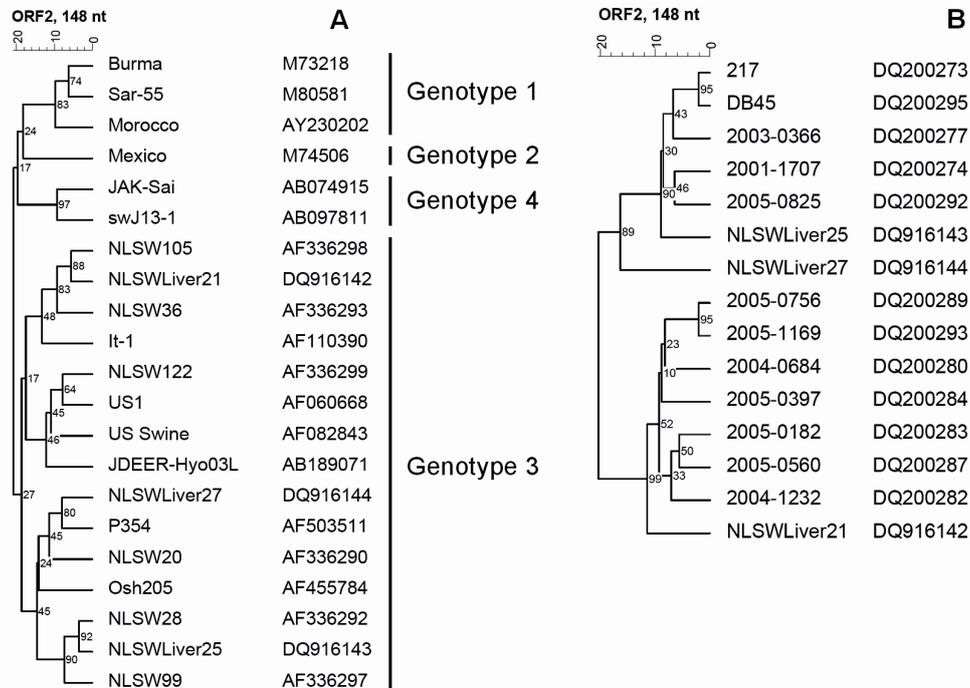
Figure 1. Rooted phylogenetic tree (UPGMA, Jukes & Cantor correction, 1,000 bootstrap replications given as percentage) for ORF2 amplicons of hepatitis E virus (148 nt). The isolates from commercial porcine livers obtained in The Netherlands were compared to local swine isolates and foreign animal and human isolates (A) or to genotype 3 isolates from patients with locally acquired Hepatitis E in the Netherlands (B). The GenBank accession numbers are indicated.

subtype: NLSWLiver21 to subtype 3e, NLSWLiver25 to subtype 3f, and NLSWLiver27 to subtype 3c. In The Netherlands, cases of locally acquired hepatitis E occur, and observed sequences showed high similarity to Dutch swine strains [57]. Therefore, sequences from commercial porcine livers were compared with all published sequences from Dutch patients with locally acquired hepatitis E between 2001 and 2005 (Fig. 1B). NLSWLiver21 showed 91% similarity with sequence 2005–1169, NLSWLiver25 showed 93% similarity with sequence 217, and NLSWLiver27 showed 88% similarity with sequence 2005–0825.

5.3.4 Experimental infection

The three extracts of the livers from which HEV sequences were obtained were inoculated in three pigs. By molecular analysis, viral RNA was detected in the high-dose control suspension, with an estimated virus concentration of 10^4 PDU/ml of inoculum. The

concentration of HEV in inocula from commercial porcine livers was estimated to be 20 PDU per inoculum. Viral excretion in feces was observed for the high-dose control animal starting at 7 dpi up to at least 16 dpi, but not at 21 dpi. None of the other four animals, including the low-dose control animal, showed viral excretion on any of the sampling occasions. No HEV RNA was observed in the liver or bile samples collected at 21 dpi for any of the five animals.

5.4 Discussion

HEV is ubiquitous in pigs worldwide, and the high similarity between human and swine HEV strains suggests transmission from swine to humans [102], from humans to swine, or from a common source to both species. In The Netherlands, HEV was detected in fecal samples from 55% of finishing pig farms [128]. Fecal excretion of virus suggested that animals were in the acute phase of infection. During the acute phase of infection, HEV replicates in the liver; therefore, the slaughter of infected pigs for consumption may result in HEV-contaminated porcine livers at retail. Hence, possible foodborne transmission of HEV may occur, but the extent will depend on the prevalence of HEV contaminated porcine livers, the number and infectivity of viral particles in livers, the effect of food preparation on HEV infectivity, the frequency of liver consumption, and the susceptibility of humans to HEV of porcine origin. HEV RNA was present in 6% of commercial porcine livers in The Netherlands. To our knowledge, this is the first study that describes the presence of HEV RNA in commercial porcine livers in a European country. In the United Kingdom, none of the 80 liver specimens from retail outlets were found to be contaminated by HEV [7]. In Japan, 2% of the pig livers were shown to contain HEV RNA [180]. In the United States, 11% of the commercial pig livers were contaminated by HEV [43]. Differences between studies may be due to a different national prevalence of HEV among pigs or to differences in detection procedures. For instance, different RT-PCR protocols may have different sensitivities, or the absence of an internal control RNA during amplification may have caused false-negative results to remain unnoticed [128].

Classification of HEV sequences was based on 148 nucleotides of ORF2, which is a relatively small, conserved region of the HEV genome but variable enough to be used for genotyping. This RT-PCR assay was chosen because it more sensitively detects HEV RNA than the nested ORF1 RT-PCR assay [128]. Amplification of a shorter fragment is generally more successful than amplification of a larger fragment when RNA is extracted from difficult matrices, such as porcine livers. We also experienced a lower sensitivity for the ORF1 RT-PCR assays, as we were unable to detect HEV RNA with a nested ORF1 RT-PCR assay in any of the commercial porcine livers that were positive by the ORF2 RT-PCR assay. The similarity between HEV sequences from porcine livers and Dutch patients was at most 93%

in the present study, and from porcine livers and Dutch swine sequences, this similarity was at most 97%. To date, 100% similarity between different HEV sequences was observed after foodborne transmission, during outbreaks with a common source, and in longitudinal samples from a single patient [141, 146]. The reported differences between HEV sequences in the present study may result from differences in time and place of isolation. Furthermore, only one identical sequence has been observed among the 42 Dutch swine sequences from different farms that are currently reported, indicating the presence of a wide variety of HEV sequences in The Netherlands [161, 128]. Therefore, the absence of 100% similarity in the present study does not indicate the absence of foodborne transmission in The Netherlands. To conclude whether the consumption of commercial porcine livers results in the foodborne transmission of HEV, further epidemiological studies or a quantitative microbiological risk assessment is required.

HEV RNA was detected only in undiluted samples in the present study, yielding an estimate of 65 PDU/g. In contrast, Japanese commercial porcine livers were positive in dilutions from 10^2 to 10^7 [180]. In our study, duplicate analysis from the positive liver with unknown sequence yielded a negative result in two of three replicates when RNA was examined. Furthermore, a new RNA extraction on a sample of this liver yielded a negative result. These findings may indicate a non-homogeneous distribution of HEV particles throughout the liver or a PDU count around the detection limit of the RT-PCR assay. This may suggest that the true prevalence is underestimated and emphasizes the need to examine multiple portions of one liver when levels of contamination are expected to be low. The relatively low count of ~65 PDU/g in the commercial livers may also reflect an end-stage of infection, considering the count of ~10^8 PDU/g for the positive control liver that was collected during the acute phase of infection. This proposed end-stage of infection may reflect the age at which animals are infected, which is thought to be between 2 and 4 months of age [147, 95]. The implication for foodborne transmission of HEV levels as reported in porcine liver cannot be assessed, because a dose-response model is not available for human HEV infections. For some viruses–for instance, for rotavirus–the probability of infection by the ingestion of one infectious viral particle is considerable [154].

In the present study, fecal HEV excretion by the pig inoculated with a high dose of HEV suggests that the experimental infection succeeded. Nonetheless, pigs that were administered a low dose of HEV or extracts of the Dutch HEV-positive commercial porcine livers did not excrete HEV in feces. In the United States, the inoculation of pigs with extracts from HEV-positive livers resulted in the fecal excretion of HEV in pigs 1 week after inoculation, indicating that livers were contaminated by infectious HEV [43]. A possible explanation for findings in the present study is that HEV RNA originated from defective viral particles. Another possible explanation is that the administered dose was too low to

cause infection. Absence of infection was observed in the low-dose control, whereas its inoculum was a dilution of the infectious inoculum given to the high-dose control that did result in the infection of a pig. Preferably, the PDU in inocula was increased, but no procedure was available to increase concentration without the risk of inactivating viral particles or without increasing the risk of causing an infarction in pigs. A dose-dependent relation for the experimental inoculation of pigs is described by Meng et al. [105]. This observation favors the hypothesis that the dose administered to pigs in the present study was too low to cause infection and that it remains unknown whether HEV-positive livers contained infectious viral particles. An alternative to the inoculation of extracts in pigs to examine the infectivity of HEV is the inoculation of susceptible cells. Two promising cell culture approaches have been described recently [38, 151].

Food preparation may have an effect on the infectivity of HEV in porcine livers. For instance, genotype 3 strains of HEV are inactivated at temperatures of 70°C and higher, whereas temperatures of 56°C, assumed to represent maximum temperatures of undercooked products, do not inactivate all viral particles [151]. Furthermore, an effect of storage in refrigerators or a freeze-thaw cycle on the infectivity of HEV is unknown. Improper cooking of food is, in general, one of the risk factors for foodborne infections [115], and because HEV is heat sensitive, it is advisable to heat porcine livers sufficiently prior to consumption, which is similar to the advice for other foodborne pathogens.

Data from a national food consumption survey in The Netherlands from 1997 to 1998 indicated that raw porcine livers were prepared by consumers on roughly 900,000 occasions annually [65]. Based on the findings in the present study (~6% positive), this amounts to about 60,000 (95% CI: 16,200 to 140,000) HEV-positive livers that are prepared annually. Because HEV is heat inactivated at ~70°C, mainly un(der)cooked livers are implicated in foodborne transmission. No data on the preparation of porcine livers are available, but data from a population-based cohort study from 1998 to 1999 showed that about 3% of the population consumes un(der)cooked pork [Van Duynhoven, pers. comm.]. Possibly, this portion of the population prefers to consume un(der)cooked pork products, including porcine livers, to maintain the tenderness of the meat, for instance. If true, the estimate of 3% may be extrapolated to livers, suggesting that about ~1,800 (95% CI: 486 to 4,200) HEV-positive un(der)cooked porcine livers are consumed annually. Further research should focus on accurate consumption and food preparation data to verify this assumption. If, indeed, the consumption of un(der)cooked porcine livers appears to be a substantial risk of HEV infection, then risk communication to the public regarding liver preparation prior to consumption can be an important preventive measure of HEV transmission via porcine liver.

Susceptibility of humans to swine HEV is suspected because of the high similarity between human and swine strains [102]. Pigs are predominantly infected by genotype 3 strains of HEV, which is similar to humans with locally acquired hepatitis E in, for example, The Netherlands [171, 57]. Twenty-three of the 25 HEV-positive porcine livers that are currently reported in Japan, the United States, and The Netherlands were contaminated by genotype 3 [180, 43]; one positive liver was contaminated by genotype 4 [180], and for one, the genotype was unknown (this study). Other data suggesting the susceptibility of humans to swine HEV include several epidemiological studies that relate direct contact with pigs to higher seroprevalence in exposed groups compared with control groups [36, 107, 175]. Cross-species infection was demonstrated by infecting primates with swine HEV [105].

In addition to a possible but unknown risk for consumers, a professional risk may be present for butchers and slaughterhouse personnel. A possible risk for contracting hepatitis E following direct contact with swine has been described, for instance, for swine veterinarians and swine farm workers [107, 175]. Especially pregnant women and people with a secondary disease have been reported to be at greater risk for an HEV infection with genotypes 1 and 4, respectively [83, 109]. Whether these properties of virulence apply to strains from genotype 3 is currently unknown, but as a precaution, appropriate awareness should be maintained when handling possibly contaminated pig products.

The probability of detecting nucleic acids is dependent on the amount of template that is analyzed and on the inhibition during amplification. Yields of RNA may differ between extraction procedures, resulting in different concentrations of template RNA and thus different detection probabilities between extraction methods. Furthermore, inhibitory factors, such as phenolic compounds, bile acids, or bile salts, are often co-extracted from difficult matrices, such as feces or liver, and may inhibit amplification [174]. A comparison of different procedures to extract RNA will identify the procedure with highest RNA yield, and the inclusion of proper internal controls and examination of RNA dilutions enable the identification of inhibition [128], as in the present study. It was demonstrated that yields of RNA differed between extraction procedures of up to 2.7 $\log_{10}$ PDU per extraction, indicating that with the RNA extraction procedure with the lowest RNA yield, no positive commercial porcine livers would have been found. Thus, the examination of environmental samples and food items that are expected to be contaminated with pathogens at low levels or that contain many inhibitory components will benefit from a comparison of RNA extraction procedures and from the inclusion of proper internal controls.

In conclusion, HEV has been detected in ~6% of commercial porcine livers in The Netherlands. Observed sequences belonged to genotype 3, which is the genotype that is associated with locally acquired hepatitis E infections in The Netherlands. A possible dose-

dependent relationship for HEV in swine was observed, because only the high-dose control inoculum resulted in the infection of a pig. Whether a public health risk due to HEV-contaminated porcine livers exists in The Netherlands is currently unknown, but data from this study can be used in a quantitative microbiological risk assessment to quantify this risk. Furthermore, areas that currently lack data to estimate the extent of a possible risk were identified. Because an infectious virus was present in commercial porcine livers in the United States [43], porcine livers should be sufficiently heated prior to consumption as a precautionary measure.

5.5   Acknowledgements

Chapter 6

Bayesian estimation of hepatitis E virus seroprevalence for populations with different exposure levels to swine in The Netherlands

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ABSTRACT

Hepatitis E virus (HEV) is ubiquitous in pigs worldwide and may be zoonotic. Previous HEV seroprevalence estimates for groups of people working with swine were higher than for control groups. However, discordance among results of anti-HEV assays means that true seroprevalence estimates, *i.e.* seroprevalence due to previous exposure to HEV, depends on choice of seroassay. We tested blood samples from three subpopulations (49 swine veterinarians, 153 non-swine veterinarians and 644 randomly selected individuals from the general population) with one IgM and two IgG ELISAs, and subsets with IgG and/or IgM Western blots. A Bayesian stochastical model was used to combine results of all assays. The model accounted for imperfection of each assay by estimating sensitivity and specificity, and accounted for dependence between serological assays. As expected, discordance among assay results occurred. Applying the model yielded seroprevalence estimates of ~11% for swine veterinarians, ~6% for non-swine veterinarians and ~2% for the general population. By combining the results of five serological assays in a Bayesian stochastical model we confirmed that exposure to swine or their environment was associated with elevated HEV seroprevalence.

6.1   Introduction

Hepatitis E virus (HEV) is an enterically transmitted RNA virus discovered in the early 1980s [176]. Since then, the virus has caused major outbreaks of hepatitis E as well as sporadic cases in humans in developing countries. A common source in epidemics is often contaminated water [39]. Mortality rates are around 1% in general [39], but may reach up to about 25% in pregnant women [83]. In addition, pre-term deliveries occur in an estimated two-thirds of HEV-infected pregnant women [83].

In developed countries, studies show seroprevalence between 0.9% and 2.6%, suggesting cases of hepatitis E occur [177]. Such cases are considered to be imported from HEV endemic areas, mainly Asia and Africa [39]. However, reports on locally acquired hepatitis E in developed countries are increasing and local sources of the virus have been identified. For instance, foodborne transmission of HEV was described in Japan, where consumption of undercooked game meat and pig livers led to clinical disease in humans [153, 180, 146]. However, no source has yet been documented for any reported locally acquired case in Europe and the United States [184, 171, 18, 93].

Possible zoonotic transmission from domestic swine to humans was suggested after the discovery of porcine HEV that showed extensive similarity to human HEV strains [106]. The possibility of inter-species transmission of HEV was corroborated by experimental infection of pigs with a human HEV strain and subsequent HEV transmission to a contact pig, and by infection of primates with porcine HEV [105]. Furthermore, direct contact with swine was suggested to be a risk factor for veterinarians and swine farm-workers due to a higher seroprevalence compared to control individuals [59, 36, 107, 175].

Several serological assays to detect HEV antigens in humans have been developed, but discordance among test results occurs when different assays are applied to the same samples [50, 96]. This makes interpretation of results difficult, especially when assays are applied to cross-sectional samples from populations and most positive results are probably from historic cases of hepatitis E. Knowing sensitivity and specificity of assays allows correction for misclassified results, but no gold standard is available to assess these two parameters. Several statistical methods are available to account for imperfect diagnostic testing in true seroprevalence estimation in the absence of a gold standard [41]. One such method estimates sensitivity and specificity of two diagnostic assays using maximum likelihood, for instance applicable for two assays used in two populations with different true seroprevalence (*i.e.* seroprevalence due to previous exposure to HEV) [64]. However, this method requires use of large sample sizes and assumes conditional independence between assays, which limits its use. A statistical approach based on Bayes' theorem is able to deal

with conditional dependence between assays and does not require large sample sizes [69]. An additional advantage of a Bayesian approach is inclusion of scientific knowledge in a probabilistic sense (designated priors).

The objective of this study was to estimate true HEV seroprevalence in three populations with differing exposure to swine, while accounting for imperfect diagnostic testing. We analyzed serum samples from swine veterinarians, non-swine veterinarians and the general population with five serological assays. Subsequently, assay results were analyzed with a Bayesian stochastical model that estimated sensitivity and specificity of each assay and accounted for potential dependency between assays.

6.2 Methods

6.2.1 Serum samples and study populations

Blood samples were collected and processed as described previously [172]. Briefly, 202 samples from veterinarians were used and a total of 648 samples from the general population were matched by gender, age and geography. Serum samples had been stored at –70 ℃ for about 2 years. Information from each veterinarian was obtained by questionnaire. Two questions addressed the relative distribution of time working with finishing and with farrowing pigs, divided in five categories: 0%, >0–25%, >25–50%, >50–75%, and >75%. Based on the estimated total time working with finishing and farrowing pigs (for quartiles, median values of categories were used for summation), veterinarians were considered swine veterinarian if >50% of their time was devoted to pigs (n=49) or non-swine veterinarians (n=153) if otherwise. Individuals from the general population were assumed to have had no professional exposure to swine.

6.2.2 Diagnostic assays

Five serological assays were used in this study: two ELISAs to detect IgG (E-1, Abbott Laboratories, Abbott Park, IL, USA; and E-2, Genelabs Diagnostics Inc., Redwood City, CA, USA), one ELISA to detect IgM (E-3, Genelabs Diagnostics), one Western blot to detect IgG (WB-1; Mikrogen, Martinsried, Germany) and one Western blot to detect IgM (WB-2; Mikrogen). All serum samples were examined with E-1, E-2 and E-3. All but two samples that were positive in at least one ELISA (63 positive samples) and two samples of which results were uncertain were blindly examined with WB-1 at the Medical University Graz in Austria (Figure 1). Among these 63 samples six were from swine veterinarians, 15 from non-swine veterinarians and 42 from individuals from the general population. In addition, 200 samples were examined with WB-1 and WB-2 at the National Institute for Public Health and
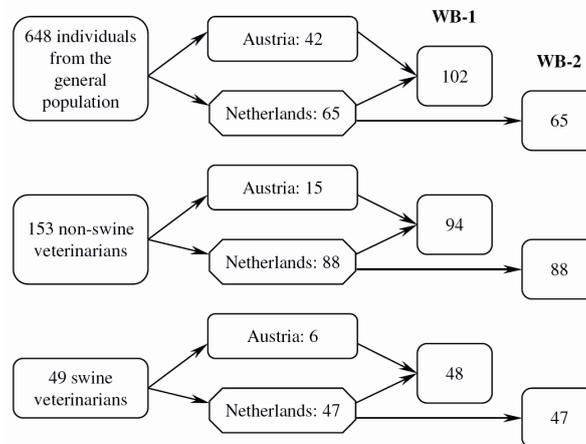
Figure 1. Test protocol for serum samples of swine veterinarians, non-swine veterinarians and individuals from the general population. All samples were examined with the two IgG and one IgM ELISAs; a selection of samples were examined in Austria and The Netherlands with WB-1 (Western blot IgG assay) and WB-2 (Western blot IgM assay).

the Environment in The Netherlands. Among these 200 samples 47 were from swine-veterinarians, 88 from randomly selected non-swine veterinarians and 65 from randomly selected individuals from the general population. In total, samples from 48 swine veterinarians, 94 non-swine veterinarians and 102 individuals from the general population were examined with WB-1.

Ratios in optical density (OD) for E-1, E-2 and E-3, and scores for WB-1 and WB-2 were calculated according to the instructions supplied by the manufacturers. Samples with an OD ratio >1 in E-2 and E-3 or an OD ratio >0.9 in E-1 were retested in duplicate. Samples were defined as positive if the average OD ratio of the duplicate test was >1 for all ELISAs. For WB-1 and WB-2, samples were defined as positive when the score was >3 (WB-1) and >5 (WB-2), as prescribed by the manufacturer. Agreement between assays was quantified with the kappa statistic [155].

6.2.3 Bayesian analysis

The Bayesian model that was applied in this study has two possible approaches for estimating sensitivity, specificity and true seroprevalence [40]. Of these two, the representation based on product conditional distributions was able to handle missing data and was therefore used. The described representation was extended to consider five diagnostic assays and three subpopulations. The model uses latent classes, which describe unknown distributions of true presence (D=1) or absence (D=0) of anti-HEV antibodies in

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serum samples. True presence or absence was assumed to be independently distributed with true seroprevalence $\pi_m=P(D=1|\text{group } m)$ among individuals sampled from subpopulation m. Hence, assay results follow a mixture of distributions for true positives and true negatives, with true seroprevalence as mixture probabilities. The Bayesian analysis was performed using the Gibbs sampler, as implemented in WinBUGS (the script can be obtained from the corresponding author) [143]. Sensitivity and specificity of each assay was assumed to be equal across subpopulations.

In Bayesian analyses, *a priori* information in a probabilistic sense (designated prior) is required for each parameter. Priors for sensitivity and specificity of each assay were based on the literature [96]. For assays based on similar antigens as E-2, sensitivity between 67% and 91% was observed. We specified a prior with a median of 50% [95% credible interval (CI) 15–98]. This prior was also used to describe specificity of all assays. Sensitivity of E-1 was considered to be lower than for E-2 based on experience, for which we specified a prior with a median of 25% (95% CI 6–66). The default prior for true seroprevalence was based on data for reported seroprevalence in industrialized countries and had a median of 12% (95% CI 0.5–51). Influence of all priors on final estimates was examined by substitution of initial priors with non-informative priors. To assess the influence of the prior for true seroprevalence, it was replaced with a less conservative prior with median 25% (95% CI 3–66) and a more conservative prior with median 7% (95% CI 0.3–31).

Differences between true seroprevalence estimates for the three subpopulations were estimated simultaneously with true seroprevalence. Statistical differences between populations were assumed present if zero was excluded from the 95% CI of the difference.

6.3   Results

6.3.1   Descriptive

Characteristics of swine veterinarians and non-swine veterinarians are shown in Table 1. Swine veterinarians were over-represented in the south of The Netherlands (χ^2, P<0.01), which reflects the concentration of swine farms in that region. Otherwise, no significant differences between swine veterinarians and non-swine veterinarians were observed. Percentages of HEV positivity in each subpopulation are illustrated per assay in Figure 2. Large differences were observed in assay outcomes (see also Table 2). Quantifying the agreement between assays yielded 'moderate' (E-1 compared to E-2: kappa ~0.5), 'slight' (E-1 or E-2 compared to WB-1: kappa ~0.15) and 'no' (E-3 compared to WB-2; kappa=0) agreement.

Table 1. Characteristics of Dutch swine (n=49) and non-swine veterinarians (n=153). The P-value relates to the chi-square test of difference between swine veterinarians and non-swine veterinarians.

Variable	Category	Swine veterinarians n*	Swine veterinarians %	Non-swine veterinarians n*	Non-swine veterinarians %	P-value
Relative time spent on	0%	0	0	73	49	-
finishing/farrowing pigs	>0% - 25%	0	0	16	10	
	>25% - 50%	0	0	64	41	
	>50% - 75%	20	41	0	0	
	>75%	29	59	0	0	
Gender	Female	6	13	34	23	0.12
	Male	42	87	115	77	
Age	<30	1	2	17	11	0.15
	30-39	14	29	34	23	
	40-49	21	44	53	35	
	≥50	12	25	47	31	
Region of practice†	North	9	18	37	25	<0.01
	Centre	16	33	72	48	
	West	2	4	14	9	
	South	22	45	27	18	
Visit to developing country	No	21	75	23	64	0.34
for more than 1 month (ever)	Yes	7	25	13	36	
Episode of diarrhoea with	None	40	82	122	80	0.77
medical consult (ever)	≥1	9	18	31	20	
Years of experience	0 - 10 years	10	20	42	27	0.79
	11 - 20 years	13	27	37	24	
	21 - 30 years	18	37	48	31	
	>30 years	8	16	27	18	

* If numbers do not add up to 49 for swine veterinarians or 153 for non-swine veterinarians, data are missing

† North: provinces Groningen, Friesland, Drenthe, Overijssel; Centre: provinces Gelderland, Utrecht, Flevoland; West: provinces Noord-Holland, Zuid-Holland; South: provinces Zeeland, Noord-Brabant, Limburg

Recently, a strategy of using combined assay results for HEV to obtain acceptable sensitivity and specificity in low-endemic areas was proposed by Herremans *et al.* [56]. In this regime, positive results with E-2 are confirmed by WB-1. Using this regime on the 200 samples that were examined with WB-1 in The Netherlands yielded seroprevalence estimates of 6.4% for the general population, 2.3% for non-swine veterinarians and 8.5% for swine veterinarians.
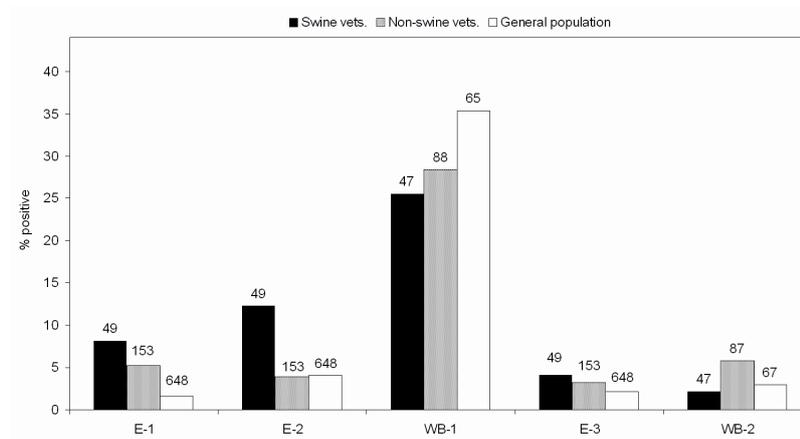
Figure 2. Percentage of HEV IgG- and IgM-positive serum samples for swine veterinarians, non-swine veterinarians and the general population, for five serological assays. Numbers above bars indicate numbers of samples from the subpopulation that were examined with the respective assay. Data on the 63 samples that were analyzed with WB-1 in Austria were omitted, because this selection was based on results for E-1 and E-2. Coding of assays is as follows: E-1, Abbott IgG; E-2, Genelabs IgG; WB-1, Westernblot IgG; E-3, Genelabs IgM; WB-2, Westernblot IgM.

6.3.2 Seroprevalence estimation

In all simulations, results from the first 4,000 iterations were discarded for burn-in. Different chain lengths (50,000–2,500,000) as well as replicate chains of equal length (50,000 and 100,000) and three parallel chains with different initial values for all parameters (chain length 50,000) were compared with respect to the stability of posterior estimates. Posterior estimates were consistent in all comparisons. The results reported in this paper were obtained by one simulation with 100,000 iterations.

Estimated sensitivity of assays varied between 10%and 63%, with wide credible intervals (Table 3). Estimated specificity of assays varied between 74% and 99%, with small credible intervals. Changing the prior for specificity affected these results minimally. Changing the prior for sensitivity affected the sensitivity estimate for E-1 most and had marginal effects on the sensitivity of other assays. True seroprevalence estimates, based on the default prior for seroprevalence, for swine veterinarians, non-swine veterinarians and the general population are shown in Table 4. The highest true seroprevalence was estimated for swine veterinarians

Table 2. Frequency counts of combined results for five serological assays against anti-HEV antibodies in three population groups differing in grade of professional exposure to swine. Other combinations than those displayed were not observed.

Test results[*]	Swine veterinarians	Non-swine veterinarians	General population	Total
− − − − −	31	56	41	128
− − − − +	1	2	0	3
− − − + −	8	16	18	42
− − + − −	1	1	0	2
− + − − −	2	1	1	4
− − − + +	0	3	1	4
− − + + −	0	1	0	1
− + − + −	0	0	2	2
+ − − + −	0	2	0	2
+ + − + −	4	1	1	6
+ + + + −	0	1	0	1
+ + − + +	0	0	1	1
+ + − + .	0	1	1	2
+ + − − .	0	0	5	5
+ − + − .	0	1	0	1
+ − − + .	0	0	1	1
+ − − − .	0	2	2	4
− + + + .	0	0	1	1
− + + − .	0	1	1	2
− + − + .	0	0	5	5
− + − − .	0	1	9	10
− − + + .	1	0	2	3
− − + − .	0	0	10	10
− − − . −	1	0	0	1
− − − . .	0	63	546	609

[*] representing: E-1, E-2, E-3, WB-1, WB-2 respectively. '-' indicates a negative test result, '+' indicates a positive test result, a dot ('.') indicates missing data.

(~11%), lowest true seroprevalence for the general population (~2%), and intermediate true seroprevalence for non-swine veterinarians (~6%). The true seroprevalence among swine veterinarians was significantly higher than among the general population, with the 95% CI of the median of the difference (0.1–24) excluding zero. The true seroprevalence among non-swine veterinarians was not significantly different from the true seroprevalence among swine veterinarians or among the general population, but a dose–response relation was suggested.

Table 3. Posterior medians and 95% credible intervals for sensitivity (SE) and specificity (SP) for five serological assays (IgG and IgM) detecting anti-HEV antibodies, based on different priors.

	Non–informative priors	Informative for SP (all)[*]	Informative for SE (IgG ELISAs)[†]	Informative for SP (all) and SE (IgG ELISAs)
Sensitivity				
E-1	62% (15%, 94%)	59% (15%, 94%)	42% (12%, 75%)	43% (14%, 74%)
E-2	63% (12%, 94%)	64% (13%, 94%)	59% (12%, 97%)	64% (15%, 97%)
E-3	16% (4%, 51%)	15% (3%, 44%)	15% (3%, 51%)	14% (3%, 42%)
WB-1	53% (14%, 87%)	51% (16%, 83%)	47% (11%, 82%)	47% (16%, 78%)
WB-2	10% (1%, 38%)	10% (1%, 36%)	9% (1%, 37%)	9% (1%, 33%)
Specificity				
E-1	99% (97%, 100%)	99% (97%, 100%)	99% (97%, 100%)	99% (97%, 100%
E-2	97% (95%, 99%)	97% (95%, 99%)	97% (95%, 99%)	98% (95%, 99%)
E-3	98% (96%, 99%)	98% (97%, 99%)	98% (96%, 99%)	98% (97%, 99%)
WB-1	74% (67%, 79%)	74% (67%, 80%)	74% (67%, 80%)	74% (67%, 80%)
WB-2	95% (92%, 98%)	96% (92%, 98%)	95% (92%, 98%)	96% (92%, 98%)

[*] For all assays, the prior emphasized a specificity of 0.75 (2·5% limit: 0·15; 97·5% limit: 0·98)
[†] For E-1 the prior emphasized a sensitivity of 0·25 (0·06; 0·66), for E-2 a sensitivity of 0·75 (0·15; 0·98)

Table 4. Posterior median (95% credible interval) for seroprevalence of anti-HEV antibodies in three Dutch subpopulations (differing in degree of exposure to swine) using different priors for sensitivity (SE) and specificity (SP).

	Non–informative	Informative on SP for all five	Informative on SE (E1 and E2)	Informative on SE (E-1 and E-2) and SP for all five
SWV[*]	10% (1%, 27%)	11% (2%, 30%)	12% (1%, 35%)	13% (3%, 36%)
NSV	5% (1%, 16%)	6% (2%, 18%)	6% (1%, 19%)	6% (1%, 21%)
GP	1% (0%, 5%)	2% (0%, 7%)	2% (0%, 8%)	3% (0%, 9%)

[*] SWV, swine veterinarians; NSV, non-swine veterinarians; GP, general population

Changing priors for sensitivity or specificity altered the true prevalence estimates to the minimum. Changing the prior for seroprevalence altered seroprevalence estimates for all groups (Table 5). The difference in true seroprevalence between swine veterinarians and the general population was 13% (95% CI 1.6–40) with use of the less conservative prior and 7% (95% CI 0.1–20) with use of the more conservative prior. Hence, the statistical difference between swine veterinarians and the general population remained. When substituting the less conservative seroprevalence prior for the default seroprevalence prior, assay sensitivity was estimated to be lower. When substituting the more conservative seroprevalence prior for the default seroprevalence prior, assay sensitivity was estimated to be higher. Estimated specificity of assays remained stable with each of the three seroprevalence priors.

Table 5. Posterior medians for seroprevalence (95% credible interval) for swine veterinarians, non-swine veterinarians and the general population, and estimated sensitivity and specificity of five serological assays, for different priors for the seroprevalence (default, less conservative and more conservative). The informative priors for specificity were used for all assays in these analyses.

	Default prior	Less conservative prior	More conservative prior
Seroprevalence estimates			
Swine vets.	11% (2%, 30%)	17% (5%, 50%)	9% (1%, 22%)
Non-swine vets.	6% (2%, 18%)	9% (3%, 34%)	5% (1%, 13%)
General population	2% (0%, 7%)	3% (1%, 16%)	2% (0%, 5%)
Sensitivity estimates			
E-1	59% (15%, 94%)	43% (10%, 88%)	64% (18%, 95%)
E-2	64% (13%, 94%)	47% (9%, 89%)	67% (15%, 94%)
E-3	15% (3%, 44%)	12% (3%, 31%)	16% (4%, 51%)
WB-1	51% (16%, 83%)	45% (15%, 74%)	53% (16%, 85%)
WB-2	10% (1%, 36%)	8% (1%, 29%)	11% (1%, 39%)
Specificity estimates			
E-1	99% (97%, 100%)	99% (97%, 100%)	99% (97%, 100%)
E-2	97% (95%, 99%)	98% (95%, 99%)	97% (95%, 99%)
E-3	98% (97%, 99%)	98% (97%, 99%)	98% (97%, 99%)
WB-1	74% (67%, 80%)	74% (67%, 81%)	74% (68%, 80%)
WB-2	96% (92%, 98%)	96% (92%, 98%)	96% (92%, 98%)

6.4 Discussion

The significant difference in estimated seroprevalence between swine veterinarians (~11%) and the general population (~2%) suggests a positive correlation between direct contact with swine, or swine farms, and seropositivity for anti-HEV antigens in humans. Our results agree with those from a US study that found 26% HEV seroprevalence for swine veterinarians compared to 18% for control subjects [107]. A similar association was observed for swine farm workers compared to control subjects in Moldova (51% compared to 25%), Taiwan (27% compared to 8%) and the United States (11% compared to 2%) [59, 36, 175]. In contrast, no difference in seroprevalence was found between pig farmers and controls in Sweden (13% vs. 9.3%, respectively) [113]. Differences between seroprevalence estimates for comparable groups have probably been caused by differences in country of origin of study populations, in the study populations themselves, and in serological assays used. All previous studies that relate contact with swine to HEV seroprevalence examined presence or absence of swine exposure. We also studied a group of individuals with less exposure to swine than swine veterinarians, but more exposure than the general population. Data tentatively suggest a positive relation between seroprevalence and level of exposure to

swine. However, as this type of study design lacks the ability for causal inference, other possible sources of HEV on swine farms cannot be excluded as a possible explanation for elevated HEV seroprevalence. Therefore, the data presented confirm that exposure to swine or the swine environment is associated with elevated HEV seroprevalence.

Veterinarians may indeed be exposed to HEV during treatment of pigs, as HEV RNA was present on at least 54% of 97 randomly selected finishing pig farms in The Netherlands in 2005 [128]. However, other farm animals, such as cattle, sheep and goats, have also been shown to carry antibodies to the virus, albeit at a lower seroprevalence than swine, and might be a source of HEV. The seroprevalence of ~6% for non-swine veterinarians compared to 2% for the general population, although not significantly different, might, next to low-level swine exposure, also be the result of direct contact with other animal sources that are susceptible to HEV. Other animals that may spread HEV though faecal deposits should be examined in more detail, preferably with molecular methods.

Discordance between results from serological assays targeting similar immunoglobulins (IgG or IgM) against HEV was observed in this study. This observation has been reported previously [50, 96] and complicates the interpretation of cross-sectional HEV seroprevalence estimates based on results from a single assay. Preferentially, true seroprevalence estimates are adjusted for sensitivity and specificity of assays [155], but true sensitivity and specificity of assays are always unknown. Relative sensitivity and specificity of assays may be estimated from sample sets obtained from humans or animals during the acute phase of infection, but assay performance will probably be different when assays are used in a cross-sectional or cohort study. For instance, levels of HEV antibodies decay in time, making discrimination between positive and negative samples more difficult [98]. Statistical modelling is useful in such cases to estimate sensitivity and specificity of assays and subsequently true seroprevalence in the absence of a gold standard, as was applied in the present study. Ideally, the approach described in the present study should always be used to account for misclassified samples in a cross-sectional or cohort study.

The estimated seroprevalence of about 2% for the general population is consistent with most findings from developed countries [177]. Previous estimates for The Netherlands include: 0.4% of 1275 blood donors [182], 0% of 50 blood donors [57] and 3.6% of 167 individuals from the general population [56]. Such differences in estimates may be explained by misclassified results, by different study populations or differences in serological assays that were used. A proposed testing regime for low-endemic countries suggests that positive results from IgG and IgM ELISAs should be confirmed with an IgG and IgM Western blot [56]. However, although the proposed testing regime may be a simpler alternative to estimate prevalence, estimates may be biased as no correction for sensitivity and specificity

is applied. Applying the proposed regime to the present data showed that seroprevalence estimates for non-swine veterinarians and the general population were overestimated, whereas the seroprevalence estimate of swine veterinarians was underestimated. Therefore, an approach as described in the present paper should always be followed.

Data from the present study did not reveal a higher number of medical consultations by swine veterinarians compared to non-swine veterinarians. One swine veterinarian did report a history of non-ABC hepatitis in the past, but results of serological assays performed on this sample in the present study were negative. The absence of a higher number of medical consultations for swine veterinarians may suggest that most encounters with HEV by veterinarians result in subclinical or mild infections.

The initial selection of 63 samples to be analyzed with WB-1 was based on results from E-1, E-2 and E-3, and such a selection may affect seroprevalence estimates. However, in a Bayesian analysis, no special provision is needed for the selection of samples for WB-1, when all available data are analyzed, because selection does not affect priors or (product conditional) likelihood. The priors and the kernels of the likelihood remain the same.

In this study, probabilities of detecting IgM and IgG were treated as if they were unrelated to the stage of the disease. However, it is known that IgM is a marker of acute infection, whereas IgG is a marker for past infections [127]. Theoretically, inclusion of IgM data may subsequently result in an underestimation of seroprevalence. However, underestimation of the presented seroprevalence due to inclusion of IgM assays was probably minimal, because sensitivity of IgM assays was low and specificity of IgG assays was high. To show that underestimation of the true seroprevalence did not occur, we repeated the analysis with data from IgG assays only. Minimal change in true seroprevalences and no change in conclusions were observed (data not shown). The advantage of including IgM assays in this study was to detect recent HEV infections for which an IgG response was still absent, and to increase statistical power.

In conclusion, discordance between results from different serological assays requires analysis of results from multiple assays to obtain seroprevalence estimates for HEV in industrialized countries. Presented data suggest an increased risk for swine veterinarians due to their professional exposure to swine or swine environments. Non-swine veterinarians, although not statistically significant, were found to also have a higher true seroprevalence estimate compared to the general population, which may be caused by exposure to swine (environments), albeit at a lower level than swine veterinarians, or due to other potential animal sources.

6.5 Acknowledgments

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Chapter 7

General discussion

General Discussion

Hepatitis E virus (HEV) infections occur in people worldwide and are increasingly detected in developed countries. In developing countries, the source of infection is mostly contaminated water [122], whereas in developed countries the source of infection is mostly unknown (reviewed in Chapter 1 of this thesis). HEV is present in swine worldwide and swine-HEV is suggested to be a source for human HEV infections in developed countries. In The Netherlands, the prevalence of HEV RNA excretion on randomly selected fattening pig farms was 55% [128]. Evidence for direct or indirect transmission from swine to humans has not yet been reported, hindering attribution of pigs as source for human HEV infections. The studies described in this thesis were conducted to clarify the transmission of HEV genotype 3 infections among pigs and to humans in the Netherlands. These studies include the estimation of the transmission potential of HEV in pigs (Chapter 2), estimation of the course of HEV-infection in pigs and identification of the potential sources leading to HEV-transmission (Chapter 3) and estimated the likelihood and contribution of faecal-oral HEV-transmission among pigs (Chapter 4). Furthermore, we assessed whether humans in The Netherlands are likely to be exposed to swine-HEV through consumption of porcine liver (Chapter 5) and through direct contact with pigs (Chapters 6). In this general discussion, the results are discussed in broader perspective.

7.1 Transmission of HEV among pigs

7.1.1 Transmission route

In Chapter 2 of this thesis it was shown that HEV spreads among pigs. Transmission of HEV among pigs is assumed to follow the faecal-oral route. We estimated in Chapter 4 that indeed faecal-oral transmission causes between 6% and 100% of the HEV infections in pigs that were observed in the transmission experiment. Hence, faeces is a likely source for HEV transmission among pigs. Furthermore, considering the variability in the estimated contribution of faeces, faeces may not account for all HEV transmission. This finding means that other sources may also contribute to HEV-transmission. Urine is the most likely candidate considering its potential HEV-titer and its volume of excretion (Chapter 3), albeit that the presence of infectious HEV in urine needs to be confirmed. In Chapter 2, the R_0 for HEV was conservatively estimated at 8.8, using an infectious period of 13 days. In Chapter 3, we provide a joint estimate for the infectious period based on data from both blocks of the transmission experiment, yielding an estimate of 23 days. The latter estimate indicates an estimated R_0 of ~15, assuming that the presence of HEV RNA in this period represents the presence of infectious HEV particles. Given the total number of about 15 newly infected pigs during a HEV-episode, then 6% of the newly infected pigs caused by faeces leads to 14 new HEV cases caused by another source, possibly urine. The estimated R_0 for HEV-transmission through urine then is 14 and urine causes in itself sufficient transmission of HEV among pigs to cause outbreaks. From the perspective of intervention measures, it presumably will not matter whether pigs are infected by excreted faeces or urine; both matrices add to the amount of HEV in the environment that may cause new infections. From a public health perspective and a virological perspective, however, the contribution of urine in the transmission process needs to be clarified.

7.1.2 Dynamics of transmission among pigs

In chapter 2 of this thesis, the basic reproduction ratio for HEV transmission among pigs was estimated conservatively at 8.8 from experimental data. This implies that the probability of onset of an epidemic is about 90% ($1-1/R_0$) when one infectious pig is introduced into a HEV-naïve population of pigs. The transmission potential of HEV among pigs on farms remains to be assessed, because the contact structure for pigs on farms differs from that in the experiment. A lower contact rate will reduce the rate of transmission (β), and therefore R_0. Furthermore, barriers between pigs may alter the intensity of the contact, which lowers the probability of a successful (*i.e.* resulting in transmission) contact and therefore lowers β and R_0.

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The R_0 may differ for different HEV strains, depending on its stability or virulence (indicated by the ability to invade the host cells (infectivity) and by the severity of disease produced). HEV strains that are more rapidly inactivated than other HEV strains, and thus have a lower stability, lead to a decrease in the concentration of infectious HEV in the environment. Therefore the probability of a successful contact per unit of time will be lower and the estimates for β and R_0 will be lower. HEV strains with a higher infectivity than other HEV strains have a greater probability of causing infection upon contact. A greater probability of infection will increase β and thus R_0. The infectivity may also relate to the severity of infection and thereby influence the rate of recovery (α; 1/length of the infectious period). A less infective HEV-strain may cause a less severe infection from which pigs recover more quickly. This results in a shorter infectious period and therefore a higher α. As R_0 equals β/α, the estimated R_0 will be lower. The experiments in Chapter 2 were all done with one HEV strain of genotype 3. With respect to intervention measures it will be important to assess any variation in virulence and inactivation among different HEV strains, because this variation may cause intervention measures to reduce R below unity for only particular HEV-strains. And in the end effective intervention measures should reduce R below unity for all HEV strains.

Immunity, maternal and acquired, means that susceptibility to infection is reduced or diminished and therefore influences HEV-transmission among pigs on farms. Maternal antibodies against HEV were observed in piglets of HEV-infected sows after birth. These antibodies disappeared between 1 to 9 weeks postpartum [106, 25], depending on the levels of antibodies received. These maternal antibodies appear to protect against HEV infection, because piglets with maternal antibodies against HEV were suggested to remain uninfected until antibodies disappeared [106]. Furthermore, acquired immunity after HEV infection likely prevents pigs from becoming reinfected directly after recovery (Chapter 2), but the duration of protective immunity is not clear. In Chapter 3, we described two *iv* inoculated pigs that showed a decrease in anti-HEV antibodies 28 days after the onset of faecal HEV RNA excretion. This finding may suggest loss of immunity in time. In contrast, two contact-infected pigs that were monitored >50 days after the onset of first faecal HEV RNA-excretion did not show a decrease in anti-HEV antibodies. Published data are contradictory on loss of immunity; some researchers observed such a loss [20, 148], whereas others did not [147, 164, 25]. Differences between the studies may be related to the age at which pigs are infected. If pigs become infected early in life, then a decrease in serum IgG-levels at the end of fattening period (*i.e.*, when pigs are about 6 months of age) is more likely to be observed than for pigs infected later in life. Another explanation for the contradiction may be a host-specific difference in immunocompetence: some pigs might develop a more protective immune response than others. If such difference exists, then some pigs might become susceptible

again (sooner) after recovery. The possibility of reinfections and the protective effect of maternal antibodies to HEV need to be identified to correctly model transmission of HEV on and between pig farms, and to evaluate effects of intervention measures.

If immunity is lost in time, then the progress of an epidemic follows a SIRS or SI(S) model rather than an SIR-model. In an SI(S) situation, at equilibrium state there will be about 10% of susceptible individuals ($1/R_0$) given an R_0 of 8.8 and therefore about 90% infectious individuals. In contrast, in a SIR model at equilibrium state again 10% would be susceptible, but of the remaining pigs only 0.3% of the total population would be infectious. The remainder of the pigs is recovered, assuming a 30-week fattening period and an infectious period of 3 weeks. Reported percentages of HEV excreting pigs at the and of the fattening period–the moment during fattening that most likely represents an equilibrium state–were 12.5% (2 out of 16 pigs) in Spain, 41% (21 of 51 pigs) on a simulated conventional pig farm in Canada, and 14% (1 of 7 pigs) and 9% (2 of 23 pigs) on two farms in Japan [111, 85, 26]. In a Dutch slaughterhouse, HEV RNA was detected in 14% (18 of 130) of individual pigs from 23 farms [Rutjes et al., unpublished data]. These percentages of HEV-infected pigs at slaughter age do not support the use of an SIS model, but rather an SIRS model which considers repeated reinfections.

7.1.3 Intervention measures

Intervention measures can be applied at different levels of the food production chain depending on their purpose. For instance, foodborne transmission of HEV to humans may be reduced by promoting to consumers sufficient heating of pork. To reduce exposure to HEV during pork production, either for veterinary health protection or for prevention of occupational exposure to swine HEV, the HEV incidence among domestic pigs can be reduced. Reduction of the HEV incidence among fattening pigs can be achieved by reduction of transmission among pigs, and by prevention of HEV introduction on pig farms.

Reduction of transmission

Reduction of transmission of HEV means that the rate of transmission is to be reduced. This reduction can–in theory–be achieved by eliminating infectious virus from the environment (decreasing the probability of successful contacts), by decreasing the host susceptibility (decreasing the probability of success upon contact), by decreasing the infectiousness of infected pigs (shorter period during which HEV can be transmitted) and by changing the contact structure on pig farms (decreasing the number of contacts per unit time).

To prevent epidemics from occurring, the reproduction ratio on pig farms should be reduced below unity [28]. For an R_0 of ~15 (Chapters 2 and 3), the rate of transmission needs

to be reduced at least 15-fold to prevent HEV transmission within pens (*i.e.*, $R_0 \leq 1$). The transmission routes for HEV on farms have not been identified to date, obscuring insight in the direction of intervention measures. HEV is excreted in faeces before antibodies are detectable in serum (Chapter 3), hampering the use of a test-and-cull and test-and-quarantine strategy. Therefore, prevention of transmission within pig farms may best be established by developing an effective vaccine that causes an immune response against HEV antigens and thereby decreases susceptibility of pigs to HEV. A vaccine is currently being evaluated for humans and tested in a clinical trial [140], but has not yet been reported to be developed for pigs.

For vaccine development, the possibility of becoming susceptible again after recovery (SIRS model) needs to be clarified. If indeed pigs become susceptible again after infection, then a vaccine that induces a non-life-lasting immunity against HEV may delay the onset of an epidemic [134]. This delay might result in a larger number of pigs experiencing an acute phase of HEV-infection at slaughter, leading to an increase of HEV contaminated meat at retail and therefore an increase in the risk of human exposure to swine HEV.

Prevention of introduction

Introduction of HEV on a farm starts with an index case (*i.e.*, the first HEV-infected pig) which acquired infection on-site or externally before arrival on the farm. The amount of HEV required to be ingested for a certain probability of HEV-introduction on farms (P_{intro}) can be estimated from the dose-response model derived in Chapter 4. Assuming one HEV-infected pig on a farm is sufficient to start an outbreak, the probability of at least one infected pig among n pigs that ingest HEV particles on a farm can be estimated using equation (1):

$$P_{intro} = 1 - (1 - (1 - e^{-0.02rD}))^n \tag{1}$$

where -r equals the infectivity of 4×10^{-7} (Chapter 4), the constant 0.02 is the most likely adjustment factor (TMF) from Chapter 4 to approach the observed transmission of Chapter 2 and D represents the ingested HEV dose. Equation (1) can be simplified into:

$$P_{intro} = 1 - (e^{-0.02rD})^n \tag{2}$$

The dose associated with a certain probability of introduction can subsequently be calculated from equation (3):

$$D = \frac{\text{Ln}\left((1 - P_{\text{intro}})^{\frac{1}{n}} \right)}{-0.02r} \quad (3)$$

Figure 2 shows the relation between the required HEV dose depending on the number of pigs ingesting HEV and the value for P_{intro}. If one pig on a farm ingests HEV, then the required dose for P_{intro}=0.95 is about 3×10^8 HEV particles. To illustrate the amount of *e.g.* faeces to be ingested, this dose can be divided by the distribution of the HEV concentration in pig faeces (Chapter 4). This procedure gives an estimated median of 1.5 g (95% interval: 15 mg – 0.46 kg) of pig faeces that needs to be ingested by a pig to cause introduction of HEV on pig farms. If 500 pigs on a farm ingest a equal HEV dose, then about 6×10^5 HEV particles are required to be ingested by each pig to have at least one infected pig with P_{intro}=0.95. This mounts to an estimated median required ingestion of 3 mg of faeces (95% interval: 0.3 mg – 0.9 g) per pig. These data suggest that HEV is relatively easily introduced on pig farms, which is expected from the high farm-level prevalence of HEV (Chapter 1).
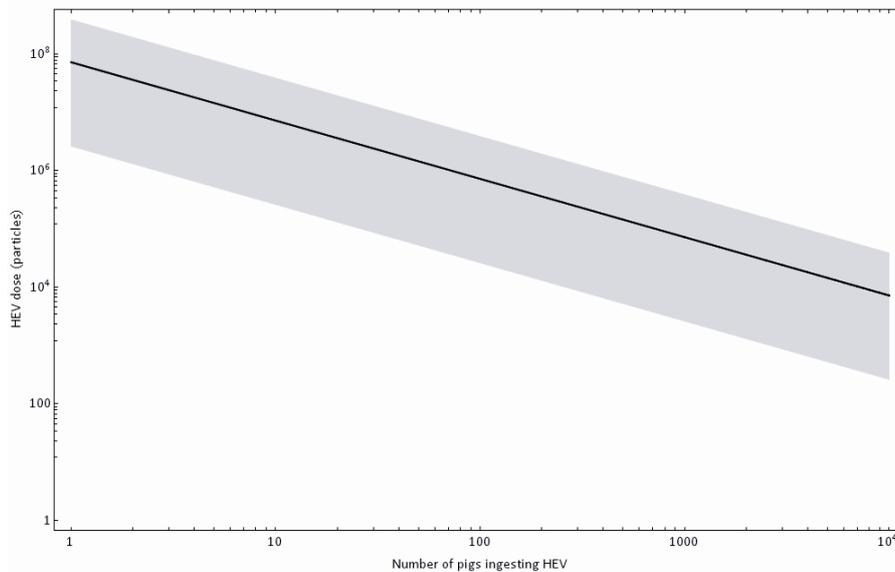
Figure 2. The estimated required HEV dose for different levels of the probability of at least one infected pig (P_{intro}) per number of pigs ingesting HEV. The grey area is the 95% interval. The black line represents the doses at P_{intro}=0.5.

A possible source for introduction during a production round is persistent HEV from previous production rounds. Thorough cleaning between production rounds can reduce the contribution of this source, as we successfully prevented HEV to persist between the two blocks of the transmission experiment (Chapter 2). However, the procedure applied during the experiment (a.o. fumigation and cleaning with high-pressure twice) is not feasible on pig farms due to high associated costs and less control of *e.g.* air flows on farms affecting the effect of fumigation. Furthermore, our procedure was not optimized to prevent HEV persistence, as these minimally required efforts are unknown. The effect of standard cleaning practices on HEV persistence could be examined on pig farms by monitoring pigs from consecutive production rounds in a longitudinal study.

Another possible source for HEV-infection in the index case is from HEV-infected sows. Faecal HEV RNA has been detected in sows of different ages [106, 20, 46, 139]. Furthermore, HEV RNA has been detected in faeces of unweaned pigs on commercial farms in Spain and the UK [46, 25, 101]. Intra-uterine transmission of HEV was not observed in an experiment with pregnant gilts [79], suggesting infected unweaned piglets acquire HEV from an environmental source. Whether HEV excreted by infected sows causes infection in piglets is unclear. HEV-infected sows provide most piglets with maternal antibodies, although some piglets from HEV-infected sows are tested negatively by serological assays [106, 79, 25, 35]. Apart from misclassification due to imperfect assay-performance, these seronegative piglets born to HEV-infected sows may be truly negative for anti-HEV antibodies and be susceptible to HEV-infection. These piglets may then acquire infection from the sow and initiate an epidemic after weaning or during the fattening phase. To understand HEV-transmission from sows to piglets, the epidemiology of HEV in piglets <10 weeks of age, including the possible sources of HEV and the transmission routes, should be known.

Wild rats (*Rattus norvegicus* and *Rattus rattus*), cattle, cats, dogs, goats, sheep and horses have been shown to carry anti-HEV antibodies in serum, which suggests exposure to HEV and possibly HEV infection [73, 42, 4, 166, 58, 158, 164, 142]. These animal species have not been examined for their transmission potential (R_0) of HEV. Wistar rats (*Rattus norvegicus*) were reported to be infected by HEV after inoculation with presumably HEV of genotype 1 (an uncharacterized Nepalese isolate) [92]. It is unknown, however, whether infected rats could infect susceptible rats, a required aspect to define rats as potential HEV-reservoir. Furthermore, it is currently unknown whether any of the potential animal reservoirs other than pigs are susceptible to HEV genotype 3. Experimental inoculation of the animal species with HEV genotype 3 and subsequent exposure of susceptible animals to the inoculated animals will be required to address this issue.

Wild boar have been found to be infected by HEV of genotype 3 in Japan, Germany, Italy, Spain and The Netherlands, and may be a potential reservoir for HEV [130, 108, 26, 74, 94]. For other viruses, such as the classical swine fever virus (CSFV), wild boar indeed are a reservoir for domestic pigs [33, 126]. In case of CSFV, transmission routes are mostly indirect and may involve *e.g.* the use of wild boar offal as feed, feeding silage from areas where wild boar range or using materials that were contaminated by wild boar carcasses [126]. HEV introduction from wild boar firstly requires that wild boar HEV strains are infectious to domestic pigs. This infectiousness is expected, because wild boar and pigs belong to the same animal species and HEV strains from both animal species are related [129]. Secondly, the net reproduction ratio (*i.e.*, the reproduction ratio for a non-fully susceptible population) for HEV-transmission among wild boar should exceed unity to be a continuous HEV source for domestic pigs. This property requires either prolonged shedding of HEV (long infectious period) or sufficient addition of susceptibles to the population. The latter is achieved by birth and possibly by regained susceptibility after infection. Interestingly, wild deer can be infected by HEV of genotype 3 in Japan [146] and The Netherlands [Rutjes et al., manuscript in preparation]. Therefore, deer, and potentially other wild animals, may contribute to maintaining HEV transmission among wild life. The subsequent likelihood of transmission to domestic pigs depends on both the frequency and intensity of contact (direct or indirect). In that context, free ranging pigs would be more likely to acquire HEV-infection from wild boar than conventionally reared pigs, due to a higher expected frequency of contact at a greater expected intensity. However, no difference was observed in anti-HEV seroprevalence among organically reared pigs (89% of 417 pigs from 43 farms), conventionally reared pigs (73% of 365 from 24 farms), and free-ranging pigs (78% of 164 pigs from 11 farms) sampled at slaughter [Reusken et al., manuscript in preparation]. These data do not support the hypothesis that free ranging pigs or organically reared pigs are more frequently HEV-infected than conventionally reared pigs. Thus far, the role of game in the epidemiology of HEV remains unclear.

Another possible route of HEV-introduction on pig farms is drinking water. In The Netherlands, 2 of 12 monthly Meuse river water samples were found to contain HEV RNA [129]. Water from the Meuse is used to produce drinking water, and although a reduction of HEV particles due to the treatment of water is expected [86], the efficiency of this reduction is unknown. Hypothetically at least, some HEV may still be present in drinking water at consumption and be a source of HEV for pigs. HEV RNA has been detected in water from a trough on two pig farms in Spain [46, 25], although the water may also be contaminated by a pig within the stable. In case of HEV-contaminated tap water humans would also be exposed. In that case, the estimated seroprevalence of 2% for the general population (Chapter 6) would be expected to be higher, unless pigs are more sensitive to HEV genotype

3 infection than humans. The latter is currently unknown. No data on HEV RNA in tap water has been published to date.

Given the estimated R_0 of 8.8 (Chapter 2), controlling the HEV incidence on fattening pig farms by minimizing the risk of introduction may be more effective than by reducing interpig transmission. However, current data are insufficient to identify the source of HEV introduction on pig farms. Data on pigs of all ages are required to explain the epidemiology of HEV on pig farms, and especially data on pigs <10 weeks of age as the index case (or cases) may well be infected in this period. A longitudinal study of a selected group of piglets on several pig farms will clarify the origin of HEV on pig farms and the possible transmission routes. In addition, such a study can support both the development and timing of implementation of intervention strategies.

7.2   Public health significance of HEV in pigs

Human exposure to swine HEV can result from direct or indirect exposure. Direct exposure is defined as exposure to an original source, *e.g.* a HEV-infected animal or its product. Indirect exposure is defined as exposure to sub sources that were contaminated from an original source, *e.g.* contaminated water from porcine manure runoff from pastures.

Direct exposure to pigs likely occurs for pigs farmers and their household members, pig transporters, veterinarians, slaughterhouse personnel and butchers, whereas the general population will experience no or occasional direct exposure to pigs. Indeed, a higher anti-HEV seroprevalence has been reported for pigs farm workers [59, 36, 175] and veterinarians [Chapter 6, 107] compared to the general population. Moreover, a slaughterhouse worker was suggested to have acquired hepatitis E after handling pig organs [116], personnel of pork companies in Taiwan showed a higher anti-HEV seroprevalence compared to the reference group [59] and a case of hepatitis E was attributed to contact with a pet-pig in France [124]. However, when retrospectively examining the HEV-origin for hospitalized human hepatitis E cases in developed countries, direct contact with pigs had occurred in only few cases [66, 109, 24, 12]. Hence, at the population-level direct exposure to pigs is likely to be of minor importance for HEV infections, at least for the severe human cases of hepatitis E that require hospitalization.

Another route for direct exposure to swine HEV is through food products. HEV meat and organs can be intrinsically contaminated by HEV RNA (Chapter 3 and 5) and may contain infectious HEV at some point during an infection. Foodborne transmission of HEV has been reported by consumption of uncooked deer meat [153], indicating the possibility of this transmission route. Foodborne transmission due to pork has not been reported to date, but

is suggested by epidemiological studies in which consumption of porcine liver or intestines was associated with human hepatitis E cases [180, 109]. To cause foodborne infection, HEV meat and liver need to contain infectious HEV, which we have not confirmed yet. In the USA, porcine liver at retail was able to infect pigs after *iv* inoculation [43]. Our inability to infect pigs via *iv* inoculation of porcine liver suspensions may have been caused by the low HEV concentration (~65 PCR detectable units per gram (Chapter 5)). The HEV-concentration in the commercial porcine livers from the American study was not reported.

Indirect exposure to swine HEV involves those routes that lead to cross-contaminated sources. Manure from pig farms is distributed on pastures and crop fields as fertilizer. If infectious pigs are present on pig farms and HEV is able to remain infectious during storage, then manure run-off into surface and ground water may result in HEV-contaminated water. In The Netherlands, river water has been found to contain HEV RNA of unknown origin [129]. For ground water, HEV may be removed or inactivated by passage through the soil, analogous to what has been determined for other viruses [135]. Similarly, for tap water, HEV may be removed by water treatment. The extent of the efficiency of treatment for HEV removal or inactivation is not reported at present, nor is the presence of HEV RNA in different sources for drinking water production. These efficiencies could be examined by choosing an adequate indicator for HEV and spiking water samples with that indicator. Subsequently, indicator concentrations in water before and after treatment could be assessed. The HEV concentration per L water can subsequently be adjusted for the estimated HEV removal due to treatment to assess the exposure of humans to HEV via tap water.

Although EU legislation prohibits the use of surface water for irrigation, non-commercial cultivators may use HEV-contaminated surface water. Especially soft fruits and vegetables are eaten mostly raw, omitting the protective effect of heating. Research whether irrigated foods are a potential vehicle for HEV-transmission is currently being conducted (see www.eurovital.org).

Another important gap in estimating the risk for foodborne HEV transmission is the lack of a dose-response model for HEV-ingestion by humans. To construct such a dose-response model, the HEV PDU count in food products is required as well as data related to infected consumers (amount consumed, frequency and timeframe of consumption, preparation of food, period between consumption and onset of symptoms). These quantitative data should ideally be collected routinely for each foodborne hepatitis E case.

Most (implied) cases of foodborne HEV transmission are reported for Japanese hepatitis E patients, possibly due to their preference of eating undercooked or uncooked meat and

organs [99]. The Dutch have a lower preference for eating raw or uncooked meats and organs. In general, food will be heated to a medium or well-done level prior to consumption. Heating of porcine liver at 56°C (a suggested medium-level internal temperature [38]) for 1 hour is insufficient to prevent foodborne transmission, as shown by inoculating pigs with heated suspensions of liver that formerly contained infectious HEV [44]. These results support the *in vitro* findings by Emerson *et al.* [38], who reported that about 50% of HEV was inactivated by heating at 56°C for 1 hour. In the latter study, heating at 60°C for 1 hour inactivated over 95% of the infectious HEV present. Considering a hypothetical HEV dose of 10^5 HEV particles, a reduction of infectious HEV particles by 95% would still give a HEV dose of 5×10^3 particles. These results indicate that sufficient heating of pork meat or products reduces the risk of foodborne transmission of HEV, whereas insufficient heating may cause foodborne HEV transmission.

In summary, no human case of hepatitis E has been directly linked to pigs. Indirect evidence, however, suggests that direct contact with pigs and consumption of un(der)cooked porcine liver are risk factors for HEV infection. The risk of foodborne HEV infection can be reduced by heating of the product and by preventing cross-contamination from utensils such as the cutting board and knife. Another potential transmission route for HEV to humans is porcine faeces that are discharged into the environment. This discharge may cause indirect human HEV-exposure via contaminated surface water or via food that is contaminated through irrigation water. The contribution of each of these potential transmission routes to human HEV exposure is currently unknown.

7.3 Overall conclusions

In conclusion, this thesis among others describes two routes of potential exposure of humans to swine HEV: direct contact with pigs and consumption of pork products. An important condition for pigs to pose a risk for humans is that HEV should spread among pigs. In Chapter 2 of this thesis it is shown that HEV indeed spreads among pigs and that pigs therefore have the intrinsic potential to be a true animal reservoir. This proof also corroborates the generally accepted hypothesis that interpig HEV-transmission causes the globally observed high HEV-prevalence among pigs, rather than continuous infection from other HEV-sources. The rapid spread of HEV also favors prevention measures for introduction of HEV on pig farms to reduce the HEV-prevalence within farms rather than measures for reduction of transmission. It is unclear, however, how HEV is introduced on pig farms. A longitudinal study in which multiple production rounds on the same farms are monitored could disclose the introduction route(s) and guide the development of intervention measures.

The course of HEV infection in contact-infected pigs was identified and shown to differ from the course following *iv* inoculation in pigs. These differences are important to account for in *e.g.* risk assessment studies that quantify pig transmission or human exposure to swine HEV. Furthermore, urine was shown to be a potential transmission route for HEV, whereas the virus is currently assumed to be transmitted faecal-orally only. We showed in a model study that it was indeed possible to relate the experimentally observed transmission to faecal-oral transmission, but also identified important data gaps that hampered attribution of the faecal oral route to all newly infected pigs. Possibly, other routes exist to explain all HEV transmission among pigs. The most likely sources, however, do include faeces and possibly urine as matrix for HEV due to the high HEV concentration in both matrices. The role of urine in HEV transmission should be further characterized. Furthermore, the associated infection dynamics, including portal of entry, are currently unknown and should be identified.

In addition to pigs, other local HEV sources in The Netherlands as well as other countries are identified (reviewed in Chapter 1). These sources include wild boar, deer, surface water and oysters. The relevance of each source for public health risk in The Netherlands is currently unknown. Prioritizing these sources by use of comparative quantitative risk assessment can guide further research and aid the assessment of the importance of domestic pigs in HEV exposure of humans in The Netherlands.

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Summary & Conclusions

Hepatitis E virus (HEV) is a causative agent of liver inflammation in humans discovered in 1980 and genetically characterized in 1990. The virus was first considered a local health hazard in developing countries and a travel-related health hazard for individuals from developed countries. However, individuals in developed countries including The Netherlands were found to acquire hepatitis E virus infections locally, indicating the existence of local sources for HEV. In 1995, the first report was published on the presence of an agent in pigs in Nepal that was detected by an RT-PCR targeting HEV. Sequence analysis of the amplicons was not performed and thus the presence of HEV remained unconfirmed. In 1997, confirmed HEV isolates were recovered from pigs in the USA and these isolates showed similar genetic and antigenic properties to isolates recovered from humans from the same region. In 2001, HEV was shown to be present on 25 of 115 pig farms in The Netherlands. In 2005, this prevalence was estimated at 55% with the increase being attributed mainly to improved diagnostics. The similarity between human and porcine HEV strains, together with successful infection of swine with human HEV and primates with porcine HEV, suggests the possibility of zoonotic transmission. The research described in the current thesis focused on the transmission of HEV among pigs, on the course of HEV-infection in pigs and on the exposure of pigs and humans to porcine HEV.

For pigs to be a true animal reservoir of HEV rather than being victim to infection from another source, HEV should spread among pigs. For pigs to be a possible source of human HEV infections, humans should also be exposed to porcine HEV. To identify the potential transmission routes, the course of infection in pigs needed to be studied, including the identification of ex- and secreta as possible sources for HEV-transmission and the period of infection during which these sources can contribute to transmission. This knowledge will be needed for the development of intervention strategies that target the reduction of the HEV incidence in pigs.

Transmission of HEV among pigs

To examine the potential of HEV to spread among pigs experimentally, 10 infection chains were started with each chain consisting of a single intravenously inoculated pig (*iv*-pig) and three in-contact pigs introduced in the chain at different moments (Chapter 2). One in-contact pig was contact-exposed to the *iv*-pig until transmission occurred. A second in-contact pig was then contact-exposed to the newly infected pig until transmission occurred and the procedure was repeated for the third in-contact pig. Transmission was considered to have occurred when HEV RNA was detected in individually collected faeces during three consecutive samplings (samplings were done every Monday, Wednesday and Friday). The results obtained led to an estimated R_0 of 8.8, with its lower confidence limit being 4 and thus excluding unity. This finding means that HEV is able to spread among pigs and confirms that pigs have the potential to be a true animal reservoir for HEV, as was hypothesized due to the high prevalence of HEV among pigs globally. The magnitude of R_0 indicates that the probability of the onset of an epidemic when one infectious pig is introduced into a naïve population of pigs is about 90%. The magnitude of R_0 also suggests that intervention measures on pig farms may best be directed at prevention of introduction of HEV rather than reduction of transmission within farms.

Course of HEV-infection in pigs

During the transmission experiment, pigs were necropsied to examine the course of infection in pigs and identify possible sources for transmission (Chapter 3). The course of HEV-infection for contact-infected pigs was characterized by faecal HEV RNA excretion that started at day 7 (95% confidence interval: day 5 – 10) postexposure and lasted 23 (19 – 28) days, by viremia that started after 13 (8 – 17) days of faecal HEV RNA excretion and lasted 11 (8 – 13) days and by antibody development that was detected after 13 (10 – 16) days of faecal HEV RNA excretion. The time until onset of viremia was significantly shorter for *iv*-pigs compared to contact-infected pigs, whereas the duration of faecal HEV RNA excretion

was significantly longer. At day 28 post infection, HEV RNA was detected less frequently in organs of contact-infected pigs compared to *iv*-pigs. Furthermore, HEV RNA was observed in several organs, including liver, and most interestingly in muscle samples that were proxies for pork at retail and in urine of contact-infected pigs. Foodborne exposure of humans to HEV through meat consumption may consequently occur. Furthermore, the finding of HEV RNA in urine suggests another possible transmission route for HEV. The assumed leading role of faeces in the transmission of HEV among pigs was estimated with a dose response model that was based on reported experimental infections of pigs (Chapter 4). Subsequently the fate of HEV in the faecal-oral pathway was modeled to estimate the daily ingested HEV dose. From this dose and the dose response model the daily infection risk was estimated at 0.85 (95% interval: 0.03 – 1). This daily risk leads to an estimated number of four new infections being caused by one infectious pig per day, or 87 newly infected pigs in 23 days (the average duration of faecal HEV RNA excretion estimated in Chapter 3). Based on the estimated rate of transmission per day from the transmission experiment (Chapter 2), the number of newly infected pigs in 23 days equaled 15. A sensitivity analysis for the model parameters showed that this estimated number of newly infected pigs was affected by the initial parameter values. Nevertheless, in all scenarios of the sensitivity analysis faeces was very likely the cause of most of the transmission of HEV among pigs.

Human exposure to porcine HEV

Human exposure to porcine HEV in The Netherlands was examined for two routes: consumption of porcine livers (Chapter 5) and direct contact with pigs (Chapter 6). For the foodborne route, 62 porcine livers were purchased at butcher shops and supermarkets. In four of sixty-two (6.5%) porcine livers HEV RNA could be detected. The exposure associated with this finding gave an estimate of about 1,800 (486 – 4,200) HEV-contaminated porcine livers to be consumed annually. For the direct contact route, serum samples from veterinarians and the general populations were examined with five serological assays. The veterinarians were classified as swine veterinarian or non-swine veterinarian based on their reported professional time spent working with pigs (>50% and ≤50%, respectively). Results of a Bayesian analysis showed that swine veterinarians had a higher estimated seroprevalence of anti-HEV antibodies compared to individuals from the general population (~11 vs. ~2%, respectively), suggesting direct contact with pigs or being on the premises of pig farms is a risk factor for HEV infection. Non-swine veterinarians had an estimated seroprevalence of ~6%, but this estimate was not believed to be significantly different from the other two estimates.

These two studies showed that humans in The Netherlands can be exposed to porcine HEV.

Implications for public health

No human case of hepatitis E has been directly linked to pigs yet. Indirect evidence, from literature and this thesis, however, suggests that direct contact with pigs and consumption of un(der)cooked porcine liver and possibly meat are risk factors for HEV infection. The risk of foodborne HEV infection can be reduced by adequate heating of the food product and by preventing cross-contamination from utensils such as the cutting board and knife. Another potential transmission route for HEV to humans is porcine faeces that are discharged into the environment. This discharge may cause indirect human HEV-exposure via contaminated surface water or via food that is contaminated through irrigation water. In addition to pigs, other local HEV sources in The Netherlands as well as other countries are identified. These sources include wild boar, deer, surface water, oysters and mussels. Note that HEV may not replicate in some of these sources and thus their role in the epidemiology of HEV is different from pigs and possibly other animals in which the virus does replicate and spreads. The relevance of each possible source for the public health risk in The Netherlands is currently unknown. Prioritizing these sources by use of comparative quantitative risk assessment can guide further research and aid the assessment of the importance of domestic pigs in HEV exposure of humans in The Netherlands.

Conclusions

The main conclusions and recommendations following from the research described in this thesis are:

- Human exposure to porcine HEV may occur in The Netherlands through consumption of porcine liver and meat, and through direct contact with pigs.

- HEV spreads among pigs and thus can persist in the domestic pig population.

- Contact-infected pigs differ from intravenously inoculated pigs with respect to the duration of faecal HEV RNA excretion, the interval between exposure and onset of viremia and the presence of HEV in organs and meat.

- Faeces are a likely source for HEV transmission among pigs.

- Urine may be another source for HEV transmission, albeit the presence of infectious HEV needs to be confirmed.

- The reproduction ratio of transmission of HEV on, and between, pig farms is unknown. A longitudinal study examining multiple consecutively-raised herds of pigs, preferably on multiple farms, is required to quantify that.

Samenvatting & Conclusies

Hepatitis E virus is een veroorzaker van leverontsteking bij mensen dat werd ontdekt in 1980 en genetisch gekarakteriseerd in 1990. Na ontdekking werd het virus gezien als lokaal probleem in ontwikkelingslanden en een reisgerelateerd probleem in ontwikkelde landen. Desondanks werden inwoners van ontwikkelde landen, waaronder Nederland, HEV-geïnfecteerd zonder buitenlandse reis te hebben gemaakt. Deze lokaal opgelopen HEV-infecties wijzen op het bestaan van lokale bronnen. In 1995 verscheen het eerste bewijs dat regulier gehouden varkens in Nepal een virus uitscheidden dat detecteerbaar was met een RT-PCR voor HEV. In 1997 is HEV uit varkens voor het eerst gekarakteriseerd in de USA. Deze HEV vertoonde dezelfde genetische en antigene eigenschappen als humane HEV-varianten uit dezelfde regio. In 2001 zijn de eerste HEV-sequenties bij Nederlandse varkens beschreven, met aantoonbare HEV in gepoolde faeces van 22% van 115 vleesvarkensbedrijven. In 2005 is deze prevalentie geschat op 55% (onder 97 vleesvarkensbedrijven). De gelijkenis tussen HEV sequenties van mensen en varkens, en experimentele infectie van varkens met humaan-HEV en apen met varkens-HEV suggereerden de mogelijkheid van zoönotische transmissie. In dit promotieonderzoek is de transmissie dynamiek van HEV bij varkens onderzocht, alsmede de potentiële blootstelling van de Nederlandse bevolking aan HEV uit varkens.

Om varkens te typeren als reservoir voor HEV en niet als slachtoffer van infecties uit andere bronnen is het nodig aan te tonen dat HEV spreidt onder varkens. En om een bron voor humans HEV infecties te zijn, is het nodig dat mensen worden blootgesteld aan HEV uit varkens. Daarvoor dienen de potentiële bronnen voor transmissie van varkens-HEV geïdentificeerd te worden, evenals de periodes gedurende een infectie waarin deze bronnen kunnen bijdragen aan transmissie. Deze informatie kan vervolgens worden gebruikt voor het opstellen van interventiemaatregelen die de incidentie van HEV bij varkens moeten reduceren.

HEV transmissie bij varkens

Om te onderzoeken of HEV spreidt onder varkens is een experiment opgezet bestaande uit 10 infectieketens. Elke keten bestond uit één intraveneus geïnfecteerd varken (*iv*-varken) en drie HEV-gevoelige varkens (contactvarkens). Aan elk van de 10 *iv*-varkens is een HEV-naïef varken blootgesteld (één-op-één blootstelling). Indien dit naïeve varken werd geïnfecteerd, werd een volgend HEV-naïef varken uit de keten aan het zojuist geïnfecteerde varken blootgesteld. Deze procedure werd herhaald indien het tweede naïeve varken was geïnfecteerd. Transmissie werd als succesvol beschouwd indien HEV aangetoond werd in faeces gedurende drie opeenvolgende monsternames. Met de data uit deze proef is the reproductieratio R_0 geschat op 8.8, met een ondergrens van 4 van het tweezijdige 95% betrouwbaarheidsinterval. Dit betekent dat HEV inderdaad kan spreiden onder varkens. De grootte van de geschatte R_0 geeft aan dat de kans op een uitbraak onder gevoelige varkens ongeveer 90% is. De grootte geeft eveneens aan dat interventiemaatregelen op varkensbedrijven het best gericht kunnen worden op voorkomen van introductie op een bedrijf in plaats van het voorkomen van verspreiding binnen bedrijven.

Verloop van een HEV infectie bij varkens

Tijdens het hierboven beschreven transmissie-experiment zijn varkens op verschillende tijdstippen na infectie voor sectie aangeboden om het infectieverloop en bronnen voor HEV transmissie te onderzoeken (Hoofdstuk 3). Gemiddeld begon faecale HEV RNA excretie 7 (95% betrouwbaarheidsinterval: 5–10) dagen na aanvang van blootstelling en duurde 23 (19–28) dagen. Viremie (HEV RNA in serum) was detecteerbaar na 13 (8–17) dagen van faecale HEV RNA uitscheiding en duurde 11 (8–13) dagen. Een immuunrespons was gemiddeld meetbaar op 13 (10–16) dagen na begin van faecale HEV RNA uitscheiding. De tijd tot HEV RNA excretie en viremie was significant korter voor intraveneus geinoculeerde varkens in vergelijking met contactgeïnfecteerde varkens, terwijl de duur van HEV RNA excretie langer was. HEV RNA werd minder vaak in organen van contactgeïnfecteerde varkens aangetoond dan in organen van *iv*-varkens op 28 dagen na infectie. Verder is HEV RNA

aangetoond in verschillende organen, waaronder de lever, in spierweefsel dat een proxy is voor varkensvlees in winkels en in urine van contactgeïnfecteerde varkens. Voedselgerelateerde blootstelling van mensen aan HEV via consumptie van varkensvlees zou daardoor voor kunnen komen. Daarnaast suggereert de aanwezigheid van HEV RNA in urine dat HEV via deze matrix overgedragen zou kunnen worden.

De faecaal-orale route wordt als belangrijkste transmissieroute van HEV gezien. De aanwezigheid van HEV RNA in urine alsmede de inefficiëntie van orale toediening van HEV in het infecteren van varkens, betwijfelen echter de leidende rol van faecale orale HEV-transmissie. Daarom is een risicoschatting gedaan voor de infectiekans voor varkens door inname van faeces als één varken in een hok met 12 varkens HEV uitscheidt (Hoofdstuk 4). Hierbij zijn op gestructureerde wijze eigen data en literatuurdata gebruikt in een zogenaamd Monte Carlo simulatie model. Uit data van verschillende infectieproeven met varkens is een dosisrespons relatie geschat, zodat de uiteindelijke geschatte ingenomen HEV-dosis via faeces kan worden vertaald in een kans op infectie. Het geschatte aantal nieuwe infecties uit het simulatiemodel is vervolgens vergeleken met het waargenomen aantal in de transmissieproef die eerder is beschreven. De gemiddelde kans op infectie per dag werd geschat op 0.85 (95% betrouwbaarheidsinterval interval: 0.03 – 1). Deze schatting leidt tot een totaal aantal nieuw geïnfecteerde varkens door één infectieus varken van 87 gedurende in infectieuze periode van 23 dagen (de duur van HEV RNA uitscheiding in faeces zoals geschat in hoofdstuk 3). Op basis van de transmissieproef met varkens uit hoofdstuk 2 en de geschatte infectieuze periode van 23 dagen (Hoofdstuk 4) werd dit aantal geschat op 15. Een gevoeligheidsanalyse voor de modelparameters toonde aan dat de uitkomst gevoelig is voor de initiële parameterwaarden, maar in elk scenario draagt de faecaal-orale route bij aan transmissie van HEV.

Humane blootstelling aan varkens-HEV

Humane blootstelling aan HEV is onderzocht voor twee routes: consumptie van varkenslever (hoofdstuk 5) en direct contact met varkens (hoofdstuk 6). Voor de voedselgerelateerde route zijn 62 varkenslevers uit slagerijen en winkels verzameld. HEV RNA is aangetoond in 4 van 62 varkenslevers (6.5%). De geschatte blootstelling van mensen geassocieerd met dit percentage positieve levers kwam uit op 1.800 (95% interval: 486 – 4.200) per jaar. Voor de blootstelling via direct contact met varkens zijn serummonsters van drie subpopulaties onderzocht: varkensdierenartsen (>50% van de werktijd besteed aan varkens), niet-varkensdierenartsen (<50% van de werktijd besteed aan varkens) en een willekeurige steekproef uit de algemene bevolking. Een statistisch model met Bayesiaanse grondslag toonde aan dat varkensdierenartsn een hogere seroprevalentie hebben dan

individuen uit de algemene bevolking (~11% vs. ~2%). Deze resultaten suggereren dat direct contact met varkens, of de omgeving van varkensbedrijven, leidt tot blootstelling van mensen aan HEV. De seroprevalentie van niet-varkensdierenartsen werd geschat op ~6%, wat statistisch niet verschillend werd bevonden van varkensdierenartsen en de algemene bevolking.

Beide studies tonen aan dat mensen in Nederland blootgesteld lijken te worden aan HEV uit varkens.

Implicaties voor de volksgezondheid

Tot op heden zijn er wereldwijd geen humane HEV infecties toegeschreven aan HEV afkomstig uit varkens. Indirecte aanwijzingen uit de vakliteratuur en dit proefschrift suggereren dat consumptie van varkenslever en –vlees en direct contact met varkens een risico vormen. Risico's van voedselgerelateerde blootstelling aan HEV kunnen worden gereduceerd door voldoende verhitting van het product en door het voorkomen van kruisbesmetting via keukengerei. En andere mogelijke transmissieroute is besmetting van omgevingsbronnen door afvoer van varkensmest (bijvoorbeeld via bemesting of lekkage). Deze afvoer kan leiden tot blootstelling aan HEV via oppervlaktewater of via voedsel dat besmet is geraakt met HEV door irrigatie. Varkens lijken echter niet het enige dierlijke reservoir voor HEV te zijn. Ook wilde zwijnen en herten kunnen aantoonbaar HEV RNA in faeces, bloed, lever of spierweefsel bevatten. De bijdrage van elk potentieel reservoir in de humane blootstelling aan HEV is momenteel onbekend. Een vergelijkende kwantitatieve risicoschatting kan hierin inzicht geven en daarmee helpen in te schatten wat de bijdrage van HEV uit varkens is.

Conclusies

De belangrijkste conclusies en aanbevelingen die zijn beschreven in dit proefschrift zijn:
- Humane blootstelling aan HEV uit varkens kan voorkomen in Nederland door de consumptie van varkenslever en vlees, en door direct contact met varkens
- HEV spreidt onder varkens en kan daarom persisteren in gedomesticeerde varkens.
- contactgeïnfecteerde varkens verschillen van intraveneus geinoculeerde varkens met betrekking tot de duur van HEV RNA uitscheiding in faeces, het interval tussen blootstelling en eerste detectie van HEV RNA in serum en de aanwezigheid van HEV RNA in organen en vlees.
- Faeces lijken een waarschijnlijke bron voor transmissie van HEV.

- Urine is een mogelijke alternatieve bron voor verspreiding van HEV, maar de infectiviteit van HEV in urine dient te worden aangetoond.
- De reproductie ratio van HEV transmissie binnen en tussen bedrijven is onbekend. Een longitudinale studie waarbij varkens worden gevolgd uit verschillende elkaar opvolgende koppels, bij voorkeur op meerdere bedrijven, is nodig om deze reproductie ratio te schatten.

Dankwoord

Zeist, zaterdag 26 september, 2009 om 16:29. Met een kopje koffie en muziek van vroeger op de achtergrond is het even genieten. Het moment is aangebroken om aan mijn dankwoord te beginnen. Zoals door velen al eerder opgemerkt: één van de meest gelezen onderdelen van een proefschrift. En ook het eindpunt van een traject waar je als verse AIO verwachtingsvol naar uitkijkt: de tijden van hectische experimenten achter de rug, de data geanalyseerd, het geheel opgeschreven en voor het grote deel gepubliceerd. Ikzelf ben–niet geheel tegen de verwachting in waarschijnlijk–erg trots op het behaalde. Maar dat is slechts ten dele mijn eigen verdienste. In dit dankwoord wil ik graag de mensen bedanken die hebben bijgedragen aan het resultaat.

In de eerste plaats is dit uiteraard Mart, mijn eerste promotor. Onnavolgbaar hoe snel jouw gedachten zich vormen. Je kritische analyses en openbarende uitleg hebben me ontzettend geholpen in het begrip van pathogeenverspreiding, modelering en interpretatie van resultaten. En daarnaast waren de door-mijn-toedoen sporadische lunches die we hadden en het gezamenlijke congresbezoek erg leuk. Jij mede hebt dit promotieonderzoek gemaakt tot wat het moet zijn: 'ter lering ende vermaak'.

Wim, mijn tweede promotor. Jij was één van de mensen met wie ik oriënterende gesprekken heb gevoerd om het onderwerp van mijn promotieonderzoek te bepalen. Er was weinig nodig om me te overtuigen dat hepatitis E virus een interessant onderwerp was. Na je vertrek bij het RIVM is het contact minder frequent geworden, maar niet minder steekhoudend. Ontzettend bedankt voor je hulp bij het concretiseren van de onderzoeksplannen en voor alle puntjes op de 'i'. Ik twijfel er niet aan dat we elkaar nog regelmatig zullen tegenkomen bij internationale projecten.

Ana Maria, na het vertrek van Wim bij het RIVM nam jij zijn rol bij het RIVM over. En met verve! Je bent met recht een aanvulling geweest in mijn promotieonderzoek. Om te beginnen door me de fijne kneepjes van het wetenschappelijk schrijven mee te geven. Daarnaast heb je me een objectiever en kritischer blik doen krijgen over mijn en andermans aanpak van onderzoek en over de interpretatie van resultaten. Dit is onontbeerlijk voor een goede onderzoeker en dat besef groeide dankzij jou met de jaren. Ontzettend bedankt! En ik hoop dat we nog vele jaren kunnen samenwerken, projecten kunnen opstarten en kunnen publiceren.

Klaas, je helpt me al een aardig tijdje op weg. Na mijn eerste colleges over kwantitatieve veterinaire epidemiologie was mijn interesse gewekt. Met je begeleiding tijdens mijn stages in de USA en Nederland, je examinering over mijn QVE-afstudeervak en je verwijzing naar een vacature bij het RIVM na mijn afstuderen heb je een aanzienlijke bijdrage geleverd aan mijn huidige loopbaan. En ik ben erg blij dat je ook aan mijn volgende stap, dit

promotieonderzoek, wilde bijdragen. Helaas ben je vanwege voor-mij-onduidelijke redenen geen officiële co-promotor, al rechtvaardigt je bijdrage een benoeming tot co-promotor zeker. Je snelle reacties en scherpe blik hebben menig hoofdstuk uit dit proefschrift verbeterd. Ontzettend bedankt voor al je inzet en ik hoop oprecht dat we in de toekomst nog vaker zullen samenwerken!

Gerard, met name in de eerste fase van dit promotieonderzoek ben je erg actief geweest. Jammergenoeg heeft de celkweek, goed voor bijna een jaar perfusies, tripsiniseren en cellen verversen, het niet gered in dit boekje. Ondanks dat was de tijd bij jou op het lab zeer waardevol en ben ik je daar zeer erkentelijk voor. Bedankt!

Rondom mijn promotie is een begeleidingscommissie gevormd om mee te denken over de inhoud en voortgang. Rob van Oosterom, Eric Evers, Peter Vesseur en later Marlies Hanssen, en Lotte Abelsma en later Eric Pierey, ontzettend bedankt voor jullie goede discussies en ideeën!

Saskia, ontzettend bedankt voor je waardevolle inbreng! We hebben heel wat uurtjes gezeten om de juiste conclusies uit mijn labresultaten te trekken. Het was (en is) erg prettig dat je deur altijd open staat en dat je altijd concrete antwoorden en oplossingen weet te geven. Ik hoop dat we nog veel gaan samenwerken, en dat minstens één paper de Lancet haalt!

Froukje, door jouw training in het labwerk werden mijn negatieve positieve controles uiteindelijk positieve positieve controles. Een hele prestatie, die waarschijnlijk erg veel geduld van je heeft geëist. Maar uiteindelijk is PCRen, blotten, hybridiseren en hier en daar cloneren een makkie geworden. Ontzettend bedankt voor je tijd, moeite, interesse en gezelligheid!

Harold, Willemijn en Manoj, drie naaste collega's die goede vrienden van me zijn geworden. Jullie technische expertise en onze discussies over de betekenis van mijn labresultaten hebben erg geholpen. Daarnaast ontzettend bedankt voor de nodige ontspanning buiten het werk om zoals het squashen, de spelletjes, een film of klussen op de moes (maar niet het priegelwerk, natuurlijk). Dat moeten we er zeker in houden!

Jack, Ciska, Arieke, Ronald, Hetty, Marjolijn, Arie, Juanita, Katsuhisa, Arno, Annemarie, Joost, Jurgen: bedankt voor de discussies over mijn resultaten tijdens onze besprekingen. Menig suggestie is uiteindelijk in dit proefschrift terechtgekomen. En Juanita, bedankt voor je gezelligheid als kamergenoot in de nadagen van mijn promotieonderzoek. Erg fijn om te kunnen ontladen over de promotieperikelen, afgewezen artikelen of onmogelijke reviews.

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En natuurlijk wil ik iedereen bij LZO bedanken, die ieder op hun manier hebben bijgedragen: met de vele vlaaien op verjaardagen, met de gezelligheid tijdens de pauzes, en niet te vergeten met het verzamelen van de varkenslevers voor hoofdstuk 5. Bedankt!

André Henken, samen hebben we tijdens mijn functioneringsgesprekken de plannen gesmeed voor dit promotieonderzoek. Ontzettend bedankt voor je inzet en hulp om het hele traject in gang te zetten.

Verder wil ik de collega's in Lelystad bedanken. Marieke van Es en Juliette Ketelaar voor hun hulp bij de monsterverwerking en celkweek. En Peter van Rossum, Norbert Stockhofe, Ad Korevaar, Klaas Jan Visser, Bernard Voorburg en de overige dierverzorgers voor hun inzet bij de organisatie of de uitvoering van de dierexperimenten.

I would like to express my appreciation to dr. Malcolm Banks and dr. Rod Card from the Veterinary Laboratories Agency in Weybridge, UK. I have greatly enjoyed the time spent in your lab to learn about, and practice with, ELISAs!

In het begin van mijn promotieonderzoek heb ik één dag in de week in Wageningen gezeten. Lisette, ontzettend bedankt voor je gezelligheid en humor daar. Ik ben blij dat we na mijn afstuderen nog regelmatig contact hebben gehouden en hoop dat we dat in de toekomst volhouden. En Marije, bedankt voor je gezelligheid en adviezen als toenmalig senior-AIO. Henk, bedankt dat je mijn laptop altijd draaiend hebt weten te houden. Nanette, bedankt voor al je hulp bij het plannen van afspraken met Mart en het regelen van zaken als een werkplek, kopieerkaart en toegangspas.

Nu ik alle werkgerelateerde mensen heb bedankt is het de beurt aan twee zeer belangrijke mensen in mijn leven. En dat zijn mijn ouders, want zij hebben onmiskenbare bijdrages aan mijn promotieonderzoek geleverd. De meest zichtbare daarvan is de prachtige voor- en achterkant van dit proefschrift die je hebt ontworpen, mam! Ik weet dat je goed bent, maar toch blijf je me telkens weer verbazen. Minder zichtbaar voor anderen waren jullie interesse in de voortgang van het onderzoek en aansporingen om de laatste loodjes niet te lang te laten liggen, wat erg stimulerend is geweest. Maar het meest belangrijke voor mij is toch wel jullie onvoorwaardelijke steun dit ik altijd heb gevoeld bij het maken van de keuzes in mijn leven. Lieve ouders, ik zeg het misschien niet vaak, maar ik heb altijd enorm gewaardeerd wat jullie voor Jeroen en mij hebben gedaan en gelaten. Ik hoop dat ik jullie met dit proefschrift een trots gevoel kan geven en een stukje van mijn waardering tastbaar kan maken.

Jeroen, grote broer. We lopen de deur niet bij elkaar plat, maar dat maakt onze broederband niet minder sterk. Ik ben erg trots op de manier waarop jij jezelf ontwikkeld hebt in je werk en als vader. Ik hoop dat je trots bent op je kleine broertje met dit proefschrift.

Maar uiteindelijk verdient er één de allergrootste erkenning en dat is *mi prinseca*. Je dacht toch niet dat je er met de zin hierboven vanaf kwam, hè? Haha. Lieve Manoj, ik ben ontzettend trots en gelukkig dat je in mijn leven bent gestapt. Al moest ik daarvoor mijn gordijntjes afknippen… Bedankt voor je onvermoeibare steun en je noodzakelijke aansporingen om zaken niet vooruit te schuiven. Met mijn impulsieve reacties is dat niet altijd even gemakkelijk voor je geweest, maar natuurlijk had je gelijk. Het zijn voor jou zware tijden geweest de afgelopen jaren. Niet alleen door mijn drukke werkzaamheden als AIO, maar ook door alles wat er in ons leventje veranderde. Tijdens mijn verdediging ontbreekt één belangrijk persoon, Monique, die eigenlijk naast je hoort te zitten. Ik hoop dat we vanaf nu een rustige tijd hebben met alleen maar leuke dingen en dat we de rest van onze levens met elkaar delen, in ons gerenoveerde paleisje, en met alles wat daarbij kan komen. Lief, je bent en blijft mijn allesje!

Curriculum Vitae

Curriculum Vitae

Martijn Bouwknegt werd op 1 augustus 1977 geboren te Groningen. Na het behalen van zijn VWO diploma in 1995 aan het Praedinius Gymnasium in Groningen verliet hij zijn ouderlijk huis om de studie Zootechniek aan de Landbouwuniversiteit Wageningen (nu Wageningen Universiteit) te beginnen. Binnen deze studie koos hij voor de specialisatie gezondheidsleer en reproductie. Tijdens zijn eerste afstudeervak bij de vakgroep 'fysiologie van mens en dier' deed hij onderzoek naar jodidedeficiënties bij de zwangere rat en haar nakomelingen. Vervolgens is hij met Eric Lensen naar Baton Rouge (Louisiana, VS) vertrokken om te onderzoeken of de lichaamsconditie van lacterende runderen haar productie en reproductie beïnvloeden. Na terugkomst begon hij aan zijn tweede afstudeervak bij de vakgroep 'kwantitatieve veterinaire epidemiologie', waarin hij risicofactoren identificeerde voor besmetting van rundvee met *E. coli* O157. Dit laatste was een samenwerking met het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) in Bilthoven en resulteerde na het afstuderen in 2000 in een onderzoekspositie bij genoemd instituut. Gedurende drie jaren heeft hij de prevalenties, en trends daarin, geschat van *Salmonella* spp., *Campylobacter* spp. en *E. coli* O157 bij landbouwhuisdieren. Daarnaast zijn verschillende studies verricht naar risicofactoren voor besmetting van landbouwhuisdieren met deze bacteriën. In deze jaren werd zijn interesse in het promotieonderzoek gewekt, hetgeen resulteerde in het in dit proefschrift beschreven onderzoek. Hij was hiervoor aangesteld bij de leerstoelgroep Kwantitatieve Veterinaire Epidemiologie van Wageningen Universiteit en gedetacheerd bij het RIVM. Sinds maart 2008 werkt hij als kwantitatief microbioloog bij het Laboratorium voor Zoönosen en Omgevingsmicrobiologie binnen het Centrum Infectieziektebestrijding van het RIVM. Hij schat in die functie onder andere de volksgezondheidsrisico's die zijn gerelateerd aan de aanwezigheid van bacteriën en virussen in het milieu.

Training

&

supervision plan

The Basic Package	year	credits *
WIAS Introduction Course, February 24th - 27th	2004	1.5
Biology underpinning animal sciences: Broaden your Horizon, November	2003	1.5
Subtotal Basic Package		**3**

Scientific Exposure (conferences, seminars and presentations)	year	credits *
International conferences		
Safepork, Verona (It), May 9th-11th	2007	0.9
COST 920 meeting Bertinoro (It), October 3rd-5th	2004	0.9
MedVetNet meeting, St. Malo (Fr), June 11th-14th	2008	1.2
Seminars and workshops		
Vereniging voor Epidemiologie en Economie, Deventer, Lelystad & Utrecht (NL)	'04, '06, '07	0.9
WIAS Science Day (NL)	2007	0.3
PhD Retreat (NL)	2004	0.3
Presentations		
COST920 meeting Bertinoro (It), oral	2004	1
Safepork 2007, Verona (It), oral	2007	1
Vereniging voor Epidemiologie en Economie, Lelystad (NL), oral	2006	1
WIAS Science Day 2007, Wageningen (NL), oral	2007	1
MedVetNet meeting, St. Malo (Fr), oral and poster	2008	1
Subtotal Scientific Exposure		**9.5**

In-Depth Studies	year	credits *
Disciplinary and interdisciplinary courses		
Tools for infectious disease epidemiology: diagnosis, modelling and risk, June 10th - 14th,P	2002	2
Animal agriculture and food safety risk analysis, September 15th - 26th	2008	2.9
Training Workshop on the use of GelCompar II and Bionumerics, April 11th & 12th	2005	0.6
Advanced statistics courses		
Statistics for the life sciences, May 30th - June 5th	2007	1.5
Designing animal experiments, September 21st - 23rd	2005	1
Subtotal In-Depth Studies		**8**

Professional Skills Support Courses	year	credits *
Techniques for writing and presenting a scientific paper, November 14th – 17th	2006	1.2

* one ECTS credit equals a study load of approximately 28 hours
P participated prior to start of the PhD programme

Professional Skills Support Courses *(continued)*	year	credits *
Course Supervising MSc thesis work, November 2nd & 3rd	2004	0.4
Writing Grant Proposals, three days in April & May	2007	1.8
Mediatraining, March 15th	2007	0.3
Subtotal Professional Skills Support Courses		**4**
Research Skills Training		
Preparing own PhD research proposal	2003	6
External training period at Veterinary Laboratories Agency (UK), July 10th - 23rd	2005	1
Subtotal Research Skills Training		**7**
Didactic Skills Training		
Supervising theses		
Two students (one MSc and one BSc)	2005	2.5
Tutorship		
'Boerderijproject'	2007	1.5
Subtotal Didactic Skills Training		**4**
Education and Training Total		**35**

* one ECTS credit equals a study load of approximately 28 hours

Notes