

Zoonotic Aspects of Hepatitis E Virus in Denmark

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Publication date:
2013

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):
Krog, J. S., Breum, S. Ø., Larsen, L. E., & Schultz, A. C. (2013). Zoonotic Aspects of Hepatitis E Virus in Denmark. Kgs. Lyngby: Technical University of Denmark (DTU).

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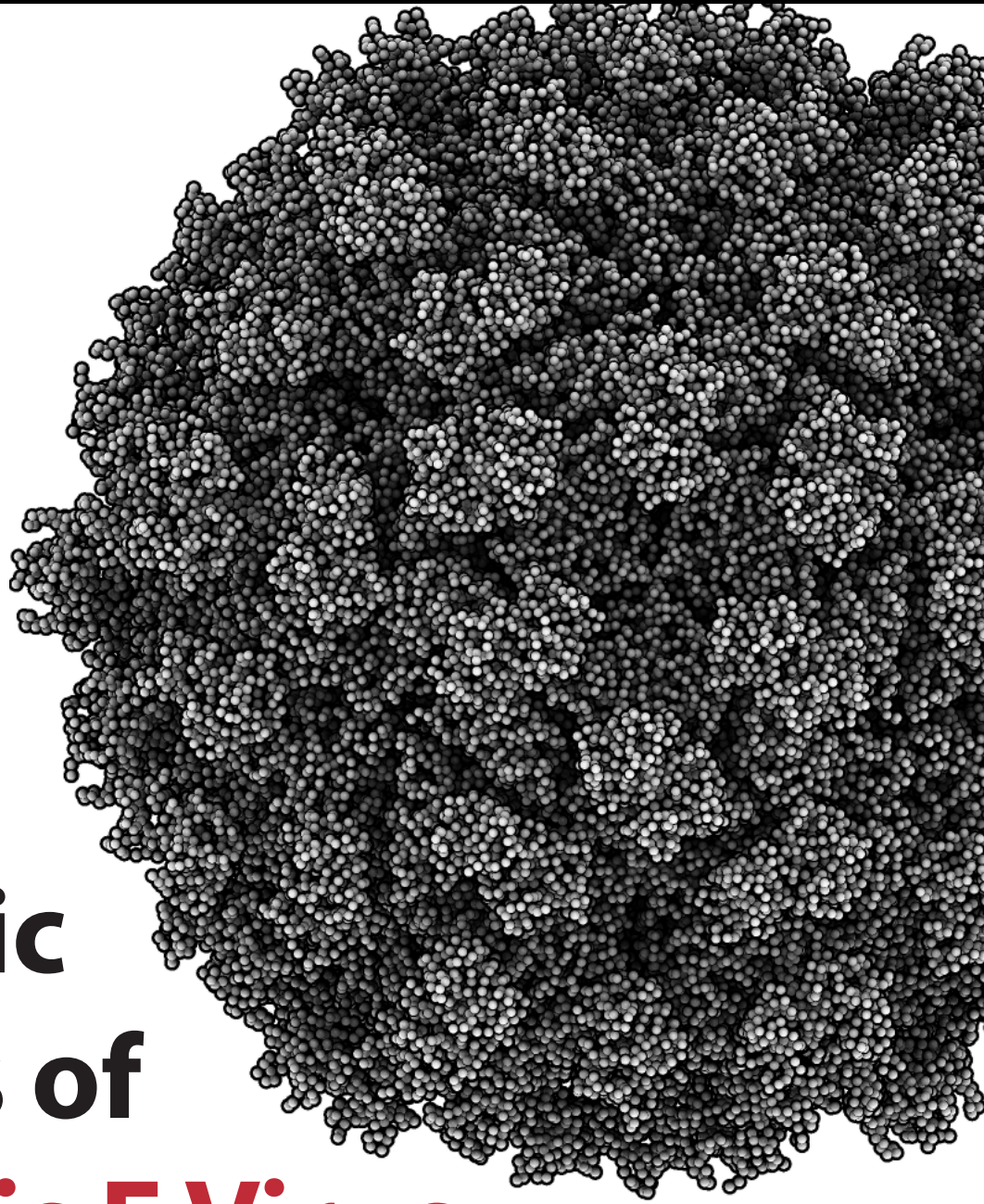
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Ph.D. Thesis, July 2013

Jesper Schak Krog, M.Sc.



Zoonotic Aspects of Hepatitis E Virus in Denmark

$$f(x+\Delta x) = \sum_{i=0}^{\infty} \frac{(\Delta x)^i}{i!} f^{(i)}(x)$$

$\int_a^b \epsilon \Theta + \Omega \int \delta e^{i\pi} = -1$

$\{2.7182818284\}$

χ^2

\approx

λ

φ ρ ε ρ τ υ θ ι ο π σ δ φ γ η ξ κ λ

1 Table of content

1	Table of content	1
2	Preface.....	4
3	Summary	6
4	Sammendrag.....	8
5	List of abbreviations.....	10
6	Objectives.....	12
7	Introduction.....	14
7.1	Historical overview	14
7.2	Diversity of HEV.....	16
7.2.1	Structure molecular properties and taxonomy	16
7.2.2	Viral entry and replication	18
7.2.3	Genotypes of HEV	19
7.2.4	Other animal specific HEV variants	22
7.3	Epidemiology of HEV	23
7.3.1	Clinical disease in humans	23
7.3.2	Clinical disease in pigs.....	27
7.3.3	Routes of HEV transmission.....	29
7.3.4	Inactivation of HEV	32
7.5	Environmental	33
7.5.1	HEV in the developed world and stability	33
7.5.2	Shellfish	35
7.6	Detection and diagnostics.....	36
7.6.1	Cell culture.....	36

Table of content

7.6.2	Real time PCR.....	38
7.6.3	ELISA	40
8	Results	42
8.1	Manuscript 1 - Detection of a hepatitis E virus variant in farmed mink in Denmark	43
8.1.1	Abstract.....	44
8.1.2	Introduction.....	44
8.1.3	The study.....	45
8.1.4	Conclusion.....	46
8.1.5	References	47
8.2	Manuscript II - Leaching of viruses naturally occurring in pig slurry to field drains and their correlation with other microorganisms	54
8.2.1	Abstract.....	55
8.2.2	Introduction.....	56
8.2.3	Materials and methods	58
8.2.4	Results	62
8.2.5	Discussion.....	65
8.2.6	Acknowledgements.....	70
8.2.7	References	71
8.3	Manuscript III - Enteric porcine viruses in farmed shellfish in Denmark	83
8.3.1	Abstract.....	84
8.3.2	Introduction.....	85
8.3.3	Material and methods.....	86
8.3.4	3 Results	88
8.3.5	Discussion.....	89
8.3.6	Acknowledgement	91
8.3.7	References	92

8.4	Manuscript IV - Hepatitis E virus – A longitudinal study from birth to consumer	100
8.4.1	Abstract.....	101
8.4.2	Introduction.....	102
8.4.3	Material and methods.....	103
8.4.4	Results.....	105
8.4.5	Discussion.....	107
8.4.6	Acknowledgements.....	109
8.4.7	References.....	109
9	Dicussion, conclusion and perspectives.....	Fejl! Bogmærke er ikke defineret.
10	References.....	121
11	Appendix I - Hepatitis E virus: En overset zoonose, der smitter fra svin.....	147
11.1.1	Fokus på HEV.....	148
11.1.2	Forårsager epidemier ved dårlig hygiejne.....	148
11.1.3	2 ud af 4 genotyper er zoonotiske.....	148
11.1.4	HEV genotype 1 og 2 er problematiske for udsatte grupper.....	149
11.1.5	HEV Genotype 3 og 4 formodes at være mindre virulente.....	149
11.1.6	HEV giver ikke sygdom hos svin.....	150
11.1.7	Smågrise smittes i 8-12 ugers alderen.....	150
11.1.8	HEV: En relativt ubeskrevet zoonose.....	150
11.1.9	HEV i rå svineprodukter gav anledning til sygdom.....	151
11.1.10	HEV i Danmark.....	151
11.1.11	HEV findes flere steder i miljøet.....	152
11.1.12	Naturligt reservoir – østers?.....	152
11.1.13	Mere forskning i smitteveje.....	152
11.1.14	Referencer.....	154

2 Preface

This PhD thesis is based on the work performed at the Technical University of Denmark (DTU), National Veterinary Institute, Frederiksberg and at the National Food Institute, Mørkhøj from June 2010 to July 2013. The work was funded by the Ministry of Food, Agriculture and Fisheries of Denmark (DFFE) (project number 3304-FVFP-09-F-011).

Writing this thesis was made possible by the support and guidance of the many fantastic people I am fortunate to be surrounded by.

First of all I would like to express my sincere gratitude to Professor Lars Erik Larsen for the opportunity to do this exciting work and for the support and guidance during the years. It has been a pleasure working with you the patience and always open door have been greatly appreciated as well as your encouragement when snow storms and technical problems made life as a scientist hard.

A special thanks goes to Dr. Solvej Østergaard Breum for mentoring me during the three years. Your continued interest and support has been invaluable to me and the time you took to guide, inspire and put things into different perspectives have helped me immensely. Thank you for caring about both the exciting work we did together on hepatitis E virus but also the well-being of those around you.

I would also like to thank Anna Charlotte Schultz for guidance and many long and beneficial talks about virus in general and all the technicalities. It has been a pleasure working with you and I am grateful for you always taking the time to make sure that I saw progress in my project.

Thanks goes also to Charlotte Hjulsager and Ramona Trebbien for support and encouragement during the years, as well as technical guidance. It has been a great pleasure getting to know and working with you.

I received an invaluable amount of help from the fantastic technical staff in the virology section at Bülowsvej. Hue Thi Thanh Tran, Kristine Vorborg and Tine Hammer made a fantastic effort to get my final analyses done in the last hectic months of my Ph.D. For that I am eternally grateful. Huge thanks also goes to Sari Dose, Helene Ringvig and Resadije Idrizi for help learning new techniques in the lab and all of you for a humorous and vibrant work environment.

During the thesis I have had the great pleasure of collaborating with some energetic and inspiring people; Anita Forslund who gave me the possibility to work with the exciting field of microbial leaching in the water environment. Trine Hammer Jensen who facilitated the work done on wild life

Preface

and farmed mink. Markku Johansen who supervised the longitudinal study. Birgitta Svensmark and Svend Haugegaard for supplying help on very short notice. Everybody in the Section for Virology with whom I have shared many inspiring hours. Thank you everybody for sharing, helping and for your interest in my project.

I would like to thank Lise Kirstine Kvisgaard and Kristina Fobian for creating a great atmosphere in our office and not keeping your tables all neat so mine looked a total mess. You have been a great support and great fun.

Lastly, my deepest thanks goes to my friends and family and especially Nadja who has set a new world record in patience and support. I look forward to returning to a life AFK and spend it with all of you.

Grøfte, July 2013

Jesper Schak Krog

3 Summary

In this thesis the epidemiology of hepatitis E virus (HEV) in Denmark was explored. Globally, four genotypes of HEV are recognized along with several species specific variants. Non-zoonotic genotypes of the virus are found in the developing world, where they cause epidemics due to fecal contaminated water resources. Zoonotic genotypes have a primary reservoir in pigs worldwide. Humans infected with HEV are often asymptomatic, but can experience an acute self-limiting hepatitis. Pigs are asymptomatic and only mild lesions have been observed in the liver of infected animals. In the developed world, sporadic cases of HEV induced disease have been more frequently reported as awareness of the disease increases, including human cases in Denmark where HEV is also prevalent in pigs.

The thesis consists of an introduction into HEV where the literature on specific subjects is reviewed. The results obtained during the work of this PhD are presented in the form of four manuscripts. Finally, the four manuscripts are discussed in a broader context.

In Manuscript I, the work performed to investigate if HEV was found in other animal reservoirs than pigs in Denmark is presented. The research focused on mink (*Nevison vison*), an economically important livestock in Denmark. The main finding was the discovery of a novel variant of HEV. The virus was found in four different locations in samples collected in 2008 and in samples collected in 2011 indicating that the virus is widespread and has been circulating in minks for years. The virus did not seem to cause clinical disease in mink, however, this should be investigated further.

Manuscript II describes the leaching of HEV along with rotavirus (RV), porcine circovirus type 2 (PCV2), somatic coliphages, *E. coli* and *Enterococcus* spp. through field soil into tile drains under natural conditions of field fertilization. The microorganisms were naturally present in the pig slurry applied to the field. The results showed that HEV along with the other five microorganisms were found in water drained from the field, indicating potential contamination of water reservoirs in connection with the untreated drainage runoff. Rotavirus was detected in deeper groundwater screen indicating the possibility of groundwater contamination of viruses originating from manure, posing a risk for the contamination of important drinking water reservoirs in Denmark.

Manuscript III focuses on the viral contamination of mussels farmed in Denmark. The mussels was assayed for HEV and Rotavirus, known to occur in shellfish and cause disease. Furthermore, PCV2 was also assayed as an indicator of porcine waste contamination. All samples analyzed were negative

Summary

for HEV and RV, however, a large proportion of the samples tested positive for the PCV2. This is the first report that shows the potential of PCV2 as an indicator organism. The lack of HEV in the mussels is in accordance with previous studies, although shellfish has been reported as the source of multiple sporadic cases of HEV infection.

Manuscript IV report the results of a longitudinal study performed in a multi-site farm, where HEV development in 104 pigs were followed from farrowing to 17 weeks of age. The pigs were divided into three groups according to the level of antibody titers of their sows. During the study, successful transfers of maternal antibodies were observed only for the pigs born to sows with the highest antibody level. Furthermore, a significant reduction of the number of pigs shedding HEV from approx. 70% to 50% was observed in the group that received maternal antibodies. Ten of the pigs that were shedding HEV at week 17 were necropsied at week 20 and three of these were still shedding HEV. Correspondingly, HEV was found in different tissues, e.g. liver, tonsils and lungs, of these three pigs. HEV was, however, not found in muscles. In addition, high level of HEV was found in 1 out of 73 Danish livers purchased at grocery stores in the larger Copenhagen area

Based on these studies it could be concluded that HEV belongs to a diverse family of viruses with variants in multiple species, and there is a possibility that many more of these will be identified during the coming years. However, clinical illness have so far only been described in humans and birds. HEV is known to be present in the water environment of poorly sanitized regions of the world, however, our study also emphasized the need for better understanding of viral leaching as well as the need for diagnostic tools enabling survey of water to maintain high drinking water quality also in the developed part of the world. We did not find HEV or RV in shellfish although indications of porcine waste contamination of shellfish occurring were observed in though the detection of the porcine specific virus PCV2. This is an interesting finding and suggest that PCV2 may be utilized to monitor viral contamination of shellfish, which is completely lacking today. Lastly, high levels of maternal antibodies can diminish the number of pigs shedding or carrying infectious HEV when entering the food supply chain. This could be utilized by vaccinating sows prior to farrowing, ensuring transfer of maternal antibodies and thereby reducing human exposure to HEV.

4 Sammenlægning

I denne PhD-afhandling undersøges tilstedeværelsen af hepatitis E virus I Danmark. Der findes fire forskellige genotyper af HEV sammen med et stadigt øgende antal artsspecifikke varianter. Ikke-zoonotiske genotyper af HEV findes i udviklings lande med lavere sanitære forhold, hvor de forårsager epidemier ved fækal kontaminering af vandforsyninger. De zoonotiske genotyper er fundet i grise fra hele verden inklusiv Danmark. Mennesker har for det meste et asymptomatisk sygdomsforløb, men det kan udvikle sig til en klinisk leverbetændelse der er akut og selvbegrænsende. Klinisk sygdom er ikke beskrevet for grise, men små læsioner på leveren er beskrevet fra grise inficeret med HEV. I de vestlige lande rapporteres der om stadigt flere sporadiske tilfælde af lokalt erhvervet HEV infektioner inklusiv i Danmark, hvor HEV også er stærkt udbredt i svinebesætningerne.

Denne afhandling indeholder en gennemgang af litteraturen om HEV for nogle udvalgte emner. Derudover bliver resultaterne præsenteret i form af fire manuskripter.

I manuskript I bliver undersøgelsen af om HEV findes I andre dyrearter end svin in Danmark beskrevet. Undersøgelsen blev fokuseret mod mink (*Neovison vison*), et økonomisk vigtigt avlsdyr i Danmark, hvor en tidligere ubeskrevet HEV variant blev fundet. De klassiske genotyper blev ikke påvist i mink. Virusset blev fundet på fire forskellige lokalisationer i prøver indsamlet i 2008 og frem til 2011 hvilket indikerer at virusset er udbredt og har cirkuleret i en længere periode. Der var ingen indikationer på at HEV varianten i mink forårsagede klinisk sygdom, men dette bør undersøges nærmere.

Manuskript II beskrives udvaskningen af HEV samt rotavirus (RV), porcine circovirus type 2 (PCV2), somatiske coliphager, *E. coli* og *Enterococcus* spp gennem jord på en mark og ud i drænvand under naturlige omstændigheder ved gødning af jorden. Mikroorganismene var naturligt tilstede i svinegyllen der blev spredt på marken. Resultaterne viste at HEV sammen med de fem andre mikroorganismer findes i drænvandet efter spredning af gylle, hvilket potentielt kan kontaminere vand reservoirer i naturen hvortil drænvandet bliver ledt ud. Rotavirus blev endvidere detekteret i nogle dybere grundvandsboringer, hvilket viste at grundvandet også var i fare for at blive kontamineret af virus. Dette kan resultere i problemer med kvaliteten af drikkevand i Danmark.

Manuskript III fokuserer på kontamineringen af muslinger dyrket til konsum I Danmark. Muslingerne blev undersøgt for tilstedeværelsen af HEV og RV, der tidlige er beskrevet i udbrud forbundet med

indtagelse af muslinger. Derudover inkluderede vi en undersøgelse for PCV2, der skulle fungere som indikator for kontaminering af svine gylle. Alle prøver blev testet negative for HEV og RV, men en stor del af prøverne var positiv for PCV2. Dette er den første undersøgelse der beskriver potentialet i at bruge PCV2 som viral indikator i skaldyr. At der ikke blev fundet HEV stemmer overens med tidligere studier i andre lande, selvom skaldyr er sat i forbindelse med flere sporadiske udbrud af HEV.

I manuscript IV præsenteres et fortløbende studie af 104 grise, hvor udviklingen af HEV blev fulgt fra farring til 17 uger. Grisene blev inddelt i tre grupper baseret på antistofniveauet af modersoen. Under forsøget blev overførsel af maternelle antistoffer observeret for grise født af søer med høje niveauer af antistoffer og en signifikant reduktion af grise der udskilte HEV fra 70% for grise uden maternelle antistoffer til 50% med maternelle antistoffer. Ti af de grise, der udskilte HEV i uge 17, blev obduceret i uge 20 og tre af grisene viste sig stadig at udskille HEV. Fra de tre grise der udskilte HEV fækalt fandtes HEV i forskellige organer f.eks. lever, mandler og lunge, men ikke i muskler og dermed kød.

Baseret på disse studier kan det konkluderes, at HEV tilhører en mangfoldig familie af virusser med varianter i mange forskellige arter, hvor der er stor mulighed for at vi kun har fundet de første. Dog har HEV kun forårsaget klinisk erkendelig sygdom i mennesker og fjerkræ. Tilstedeværelsen af HEV i vandmiljøet er velbeskrevet i lande med dårlige sanitære forhold. Vores studie viser vigtigheden af en øget forståelse af de processer, der foregår når virus udvaskes gennem jord, samt at kontrol med vores drikkevandsressourcer med henblik på virus, kan blive nødvendigt for at sikre en fortsat høj drikkevandskvalitet. Vi fandt ingen tegn på HEV og RV i skaldyr, men fandt dog tegn på fækalt kontaminering med svine gylle. Dette kan udnyttes fremadrettet til at overvåge skaldyrsområderne i Danmark og sætte mere fokus på viral kontrol af skaldyr, hvilket endnu ikke er implementeret. For at mindske eksponeringen af befolkningen for HEV vil en begrænsning af de svin der indgår i fødevarerproduktionen have en stor effekt. Vores resultater indikerer at man kan gøre netop dette ved at vaccinere søer og derved bringe deres HEV antistof niveau op.

I appendix 1 (Sektion 12) findes en dansk oversigtartikel der forklarer nogle af de zoonotiske aspekter.

5 List of abbreviations

aa – Amino acids

Ab – Antibodies

aHEV – Avian hepatitis E virus

ALT - Alanine aminotransferase

BLSV - Big liver and spleen virus

ET - Environmental temperature

G# - Genotype

HAV – Hepatitis A virus

HEV – Hepatitis E virus

HVR – Hypervariable region

NoV - Norovirus

nPCR – Nested polymerase chain reaction

NS – Non-structural

nt – nucleotides

OECD - Organisation for Economic Co-operation and Development

ORF – Open reading frame

PBS – Phosphate-buffered saline solution

PCR - Polymerase chain reaction

Poly(A) - Polyadenolated

RdRp - RNA-dependent RNA polymerase

RT-PCR – Reverse transcriptase polymerase chain reaction

RV – Rotavirus

List of abbreviations

SPF – Specific pathogen free

VLP - Virus like particle

WHO - World Health Organization

WWTP - Wastewater treatment plants

6 Objectives

With the relatively recent discovery of hepatitis E virus in pigs world wide and the realization that it is highly prevalent, concern of the implications for public health has emerged. This tendency has grown during recent years as awareness of the virus has led to an increasing number of reports of human cases. Denmark has a large production of pigs that are known to be HEV positive. At the same time the human population of Denmark have one of the highest seroprevalences in Europe. However, the transmission of HEV to humans are not fully understood. There are reports of zoonotic transmission of HEV from ingestion of un(der)cooked pork meat or liver. To what extent the population is exposed to this threat is unclear. Nevertheless, pig slurry is applied on an area that correspond to 60% of the entire area of Denmark. HEV is excreted fecally hence the pig slurry is bound to contain HEV. Furthermore reports of other animal reservoirs than pigs have emerged such as roe-deer which is also present in Denmark.

Environmental factors in the developed world has largely been overlooked or limited to studies of presence of HEV in wastewater, even though the primary route of infection in the developing world

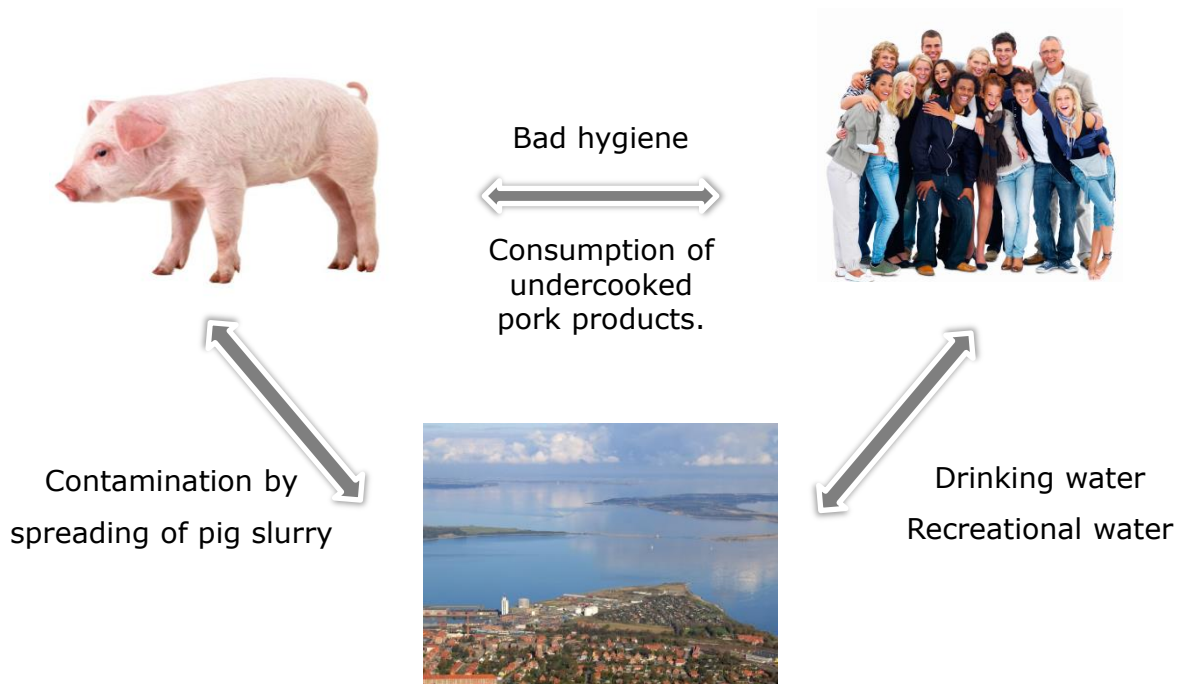


Figure 1 A schematic representation of the transmission possibilities in Denmark. The pig represent the large industry present in Denmark, which is in contact with the human population via direct contact but also as food. Here hygiene both when in contact but also in the kitchen as pork product consumer is important. Humans are also carriers of HEV like pigs. Human waste water may contain HEV and is a possible route of environmental contamination as are the application of pig slurry.

Objectives

is indeed through environmental factors such as contamination of drinking water. The tentative routes of transmission of HEV in Denmark is outlined in Figure 1. To uncover all the aspects of these transmission routes would be a daunting task.

In this study we take a new approach to uncovering the possible environmental aspects of HEV transmission in the developed world, as well as exposing alternative reservoirs of HEV. Furthermore the study of infection dynamics in pig herds with focus on prevention of human exposure was performed. The awareness of HEV is increasing but it is still evaluated to be underdiagnosed. The implications of HEV infections in the developed world are therefore largely unknown. However, HEV has proven to be a serious threat to a small part of the population that are immunosuppressed. Therefore further knowledge regarding transmission and prevention are needed to control this zoonotic pathogen.

7 Introduction

7.1 Historical overview

Hepatitis E virus (HEV) is an RNA virus with a positive sense single stranded genome. It has primarily been linked to human epidemics in India (Chobe et al., 1997; Naik et al., 1992), Pakistan (Rab et al., 1997) and China (Zhuang et al., 1991). It was first recognized as a novel pathogen during the Kashmir Valley epidemics in 1978, but the causal virus was not isolated and the conditions were therefore termed non-A non-B hepatitis (Khuroo, 1980). HEV has also retrospectively been traced back to large outbreak in Delhi, India (Viswanathan, 1957). Later, HEV was isolated following a non-A, non-B hepatitis outbreak from August to September 1981 which occurred in a Soviet military camp located in Afghanistan, the epidemic resembled the Kashmir epidemics three years before. Dr. Balayan isolated the virus from fecal samples pooled from the patients and suspended the virus into a watery phase. He then drank the sample and developed hepatitis 36 days after inoculation (Balayan et al., 1983). The primary route of transmission during most epidemics are by the fecal oral route where drinking water has been contaminated by human waste (Ashbolt, 2004; Purcell and Emerson, 2008; Teshale et al., 2010c). Because of the outbreaks occurring in poorly sanitized regions of the world, the developed part of the world has long regarded HEV as a travel related illness. In 1997 it was discovered that HEV was prevalent in American domesticated pigs (Meng et al., 1997). To reveal if pigs were susceptible to HEV isolated from humans, experimental infections in pigs were performed with HEV isolated from humans but these attempts failed (Meng et al., 1998a). This was later explained by the presence of four different genotypes of HEV; Genotype 1-4 (G1-G4). G1 and G2 has only been observed in human cases, and failed to infect the pigs, whereas G3 and G4 are zoonotic, with sporadic human cases, but the primary reservoir in pigs. Further studies have shown that G3 is found in pigs worldwide including Denmark (Breum et al., 2010). In the work by Breum et al. (2010) it was concluded that at least one of three tested sows had seroconverted in 92 % of Danish herds, and that almost half the pigs aged 9 – 22 weeks excreted HEV through feces. The Danish human population was investigated by Christensen et al. (2008), who found antibodies (Ab) against HEV in 20.8% of serum samples collected from blood donors in 2003. They also described a higher titer in farmers (50.3%) than blood donors (31.5%) in a retrospective study using samples from 1983. Locally acquired HEV in developed countries have recently been reported on numerous occasions (Colson et al., 2010; Dalton et al., 2007; Mansuy et al., 2004; Widdowson et al., 2003). The transmission of HEV is still unclear in the developed part of the world, however, there are well

Introduction

documented cases of ingestion of HEV contaminated pork products that led to HEV infections. The high prevalence of HEV antibodies suggests that alternative route of transmission exists.

7.2 Diversity of HEV

7.2.1 Structure molecular properties and taxonomy

The HEV genome of approximately 7.6 kb was first isolated in 1990 (Reyes et al., 1990). The size of the icosahedral viral particles is between 27 and 34 nm (Bradley and Balayan, 1988; Bradley et al., 1987). The virus is non-enveloped and based on these first findings of morphology and the physiochemical properties, it was placed in the family calcivirus. Later, it was discovered that the order of the non-structural (NS) internal motifs in open reading frame (ORF) 1 and the presence of a 5' m7G cap was different from calciviruses (Kabrane-Lazizi et al., 1999b; Tam et al., 1991), and any other established family of viruses. Thus, a novel family, *Hepeviridae*, was formed and HEV was placed in the genus hepevirus (International Committee on Taxonomy of Viruses (ICTV), 2009).

The HEV genome is capped at the 5' end followed by a small untranslated region (UTR) of 27 nucleotides (nt) and polyadenylated (poly(A), 150 -200 nt) at the 3' end preceded by another UTR of 65 nt (Tam et al., 1991) (Figure 2). It is divided into three open reading frames (ORF). ORF1 encodes a NS poly protein encompassing motifs identified as methyltransferase, Y domain, papain-like cysteine protease, proline rich region which might function as a hinge (Ahmad et al., 2011) and also encompasses a hyper variable region (HVR), an X domain, an RNA helicase and the RNA-dependent RNA polymerase (RdRp) (Figure 2) (Koonin et al., 1992). ORF2 (capsid protein) and ORF3 (phosphoprotein) is translated from a capped subgenomic fragment of 2.2 kb transcribed from the

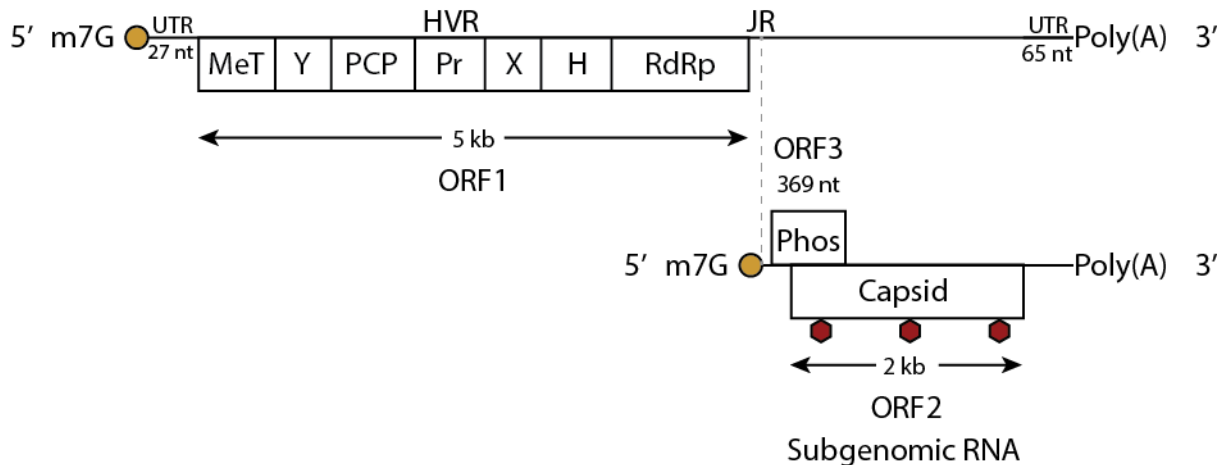



Figure 2: Schematic drawing of the HEV genome. MeT: Methyltransferase, Y: domain Y, PCP: Papain-like cysteine protease, Pr: Proline rich region overlapping with the hyper variable region (HVR), X: Domain X, H: RNA helicase, RdRp: RNA-dependant RNA polymerase, Phos:Phosphoprotein JR: Junction region. Glycosylation sites are indicated by  The 5' untranslated region (UTR) is 27 nt and the 3' UTR is 65 nt long. The sub-genomic RNA is transcribed from the intermediate negative strand viral genome copy synthesized by the RdRp.

region spanning the poly(A) tail to the junction region between ORF1 and ORF3 (Graff et al., 2006; Ichiyama et al., 2009).

The methyltransferase performs the capping of the 5' end of the viral genome (Magden et al., 2001). The most probable function of the PCP is processing of the 192 kDa poly protein, however early reports were conflicting as to if processing of the ORF1 polyprotein actually occurred. Ropp et al. (2000) described processed products of 78 and 107 kDa when ORF1 was expressed in mammalian cells, whereas Ansari et al. (2000) did not see any processing when expressed in *E. Coli* or HepG2 cell line. More recently, processing was observed and substantiated by showing that the processing could be abolished by the addition of cysteine protease inhibitors (Sehgal et al., 2006). The RNA helicase and RdRp are the core elements of the replication machinery with the helicase unwinding the RNA thereby making it accessible to the RdRp (Kadaré and Haenni, 1997). The RdRp has been shown to bind to the 3' end of the HEV genome and be able to synthesize complete complementary strands in vitro (Agrawal et al., 2001).

The 660 aa capsid protein encoded by ORF2 is the sole component of the HEV capsid. The capsid protein have been shown to assemble into two different forms of virus like particles (VLPs) T=1 and T=3 (Li et al., 2005; Xing et al., 2010), comprising of 60 and 180 monomer capsid proteins respectively (Figure 3). It is believed that the T=3 is the native capsid assembly form (Cao and Meng, 2012). The ORF2 protein contains multiple immunogenic sites and neutralizing antibodies are directed against it (Meng et al., 2001; Schofield et al., 2000; Zhou et al., 2004). The essential region

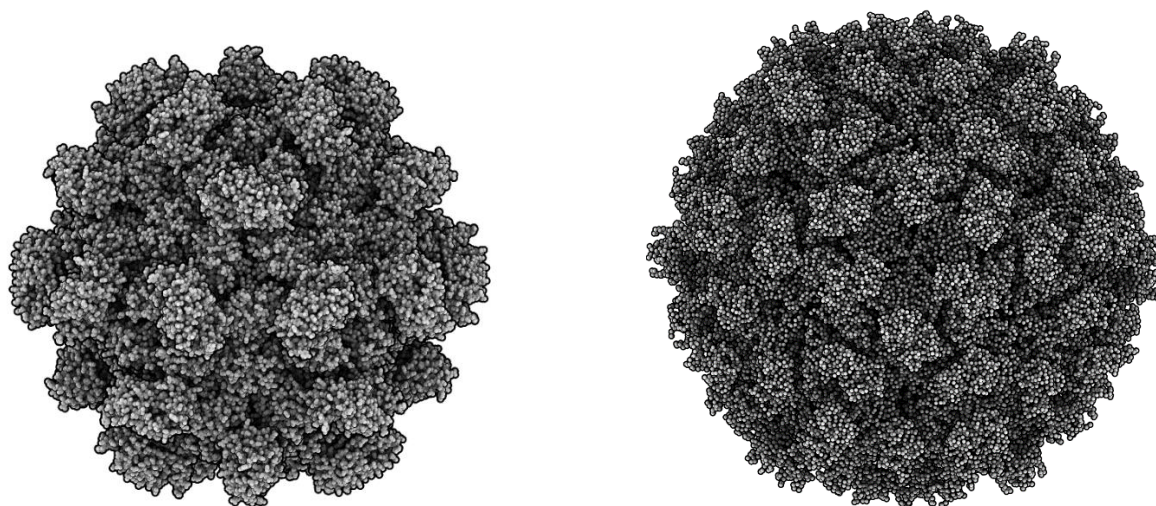


Figure 3: Structural representation of T=1 (left) and T=3 (Right) The graphics was produced using protein data bank files 3HAG (left) (Guu et al., 2009) 3IYO (right) (Xing et al., 2010) using QuteMol (Tarini et al., 2006).

in the protein for immunogenicity is aa452-aa617 and the neutralizing epitopes have recently been shown to be conformational (Zhang et al., 2008). The capsid protein has three potential glycosylation sites (Zafrullah et al., 1999). Initial studies showed that glycosylation of HEV prohibited formation of particles (Torresi et al., 1999), however, mutagenetic studies have shown that the conserved glycosylation sites are important for the formation of viable virus particles (Graff et al., 2008).

The role of the ORF3 protein, referred to as the phosphoprotein, is undetermined but multiple functions have been proposed. The protein has been localized to the cytoskeleton and functions as regulatory protein of virion assembly has been proposed (Tyagi et al., 2002). The ORF3 protein was not essential for infection of Huh7 cells (Emerson et al., 2006b), however, release of virions from cells were not observed when ORF3 was missing (Emerson et al., 2010; Yamada et al., 2009).

7.2.2 Viral entry and replication

Due to the lack of efficient cell culture systems and small animal models, the knowledge about the HEV life cycle is limited (Chandra et al., 2008). There have however been some proposed mechanism of cell attachment and the mechanism of replication. The binding of ORF2 proteins, expressed in insect cells or *E. coli*, to a subclone of Huh-7 cells were linked to the heat shock cognate protein 70 (HSC70), which is located in both cytosol but also present on the surface of Huh-7 cells (Figure 4). Similarly, HEV ORF2 has been shown to bind to heparan sulfate proteoglycans (HSPGs), more specifically the syndicans of the cell surface. By depleting the cells of specific syndicans by RNAi, binding of ORF2 was reduced and so was infectivity (Kalia et al., 2009). The HSPGs have previously been linked to multiple other viruses as receptors for viral entry (Kalia et al., 2009). Once inside the host cell, internal trafficking of HEV has been proposed to be performed by heat shock protein 90 (HSP90). In an experiment using a HEV virus like particle (VLP), entry of the VLP and relocation was observed in HepG2 cells, but only entry of the VLP and no relocation was observed when HSP90 was specifically inhibited (Zheng et al., 2010). Following uncoating, translation of ORF1 is initiated by the ribosome and Transcription of a negative strand intermediate of the viral genome is made by the RdRp. From this, full genome copies of the HEV genome is made for progeny viral particles as well as the subgenomic RNA used for the translation of ORF2 and ORF3 proteins important for viral assembly and egression.

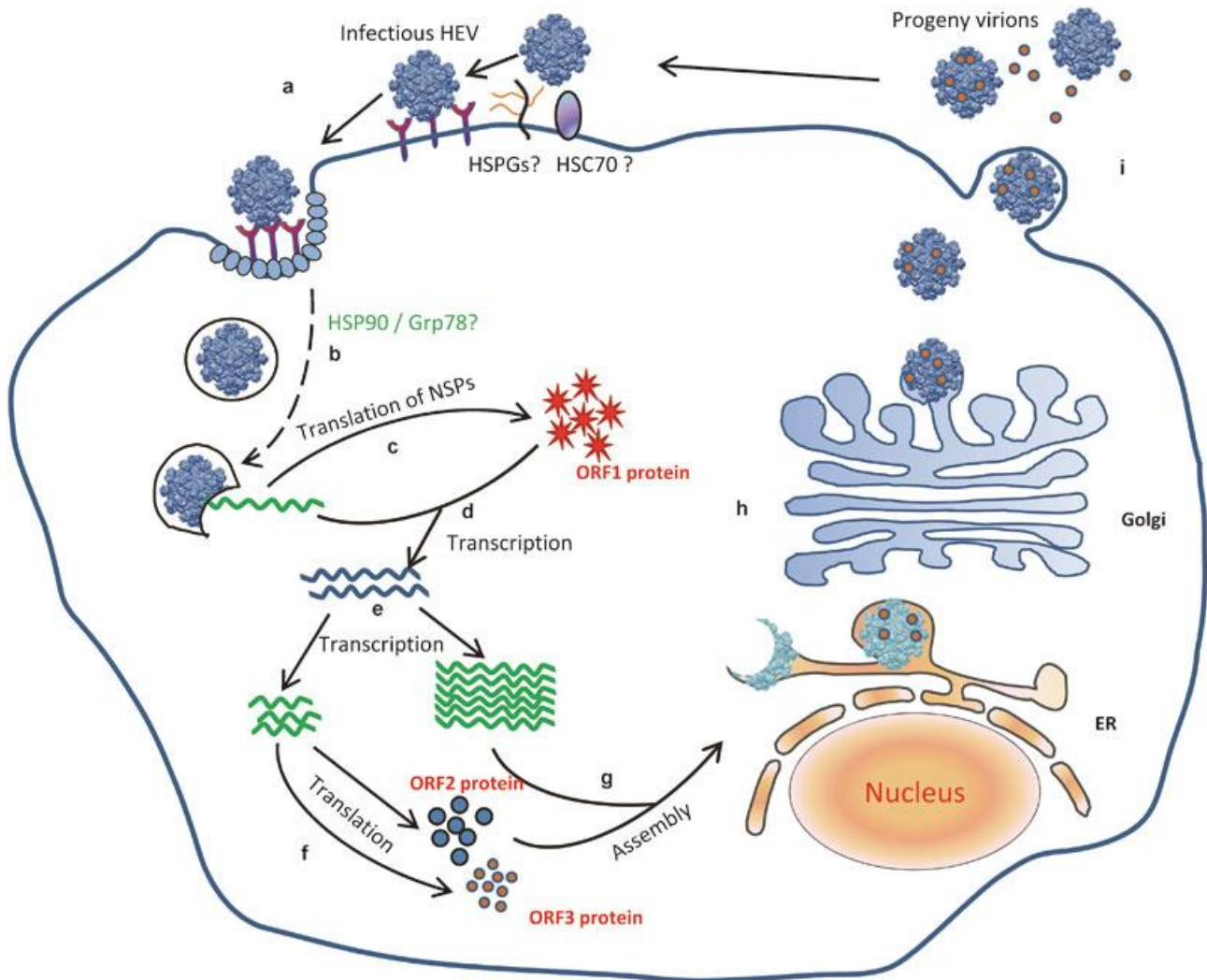


Figure 4: Viral entry and replication of HEV. (A) Binding and internalization of virus. (B) Intracellular transportation of the viral capsid and unpacking. (C) Transcription of NSP (ORF1) from the released viral genome. (D) Transcription of negative strand complementary viral genome. (E) Transcription of the negative sense RNA into sub genomic RNA and new viral genomes to be packed in progeny viral particles. (F) Translation of sub genomic RNA into capsid protein (ORF2) and phosphoprotein (ORF3). (G) Assembly of progeny viral particles (H) Newly packed virions are transported to cell the membrane aided by the ORF3 protein. (I) release of virions. From Cao and Meng (2012)

7.2.3 Genotypes of HEV

There are four different genotypes of HEV that infects humans (Schlauder and Mushahwar, 2001) of which two is zoonotic (Cooper et al., 2005; Feagins et al., 2008b; Halbur et al., 2001; Meng et al., 1998b). G1 is the most frequently reported human genotype, whereas G2, which is mostly observed in sub-Saharan Africa and Mexico is rarely reported. G1 and G2 are mostly seen in developing countries and are the cause of larger outbreaks and epidemics. However, G1 was reported to occasionally circulate in Spain (Clemente-Casares et al., 2009). Genotype 3 is zoonotic and found in

pig populations world-wide and causes sporadic autochthonous incidents in humans in the western world (van der Poel et al., 2001). Genotype 4 is also zoonotic and predominantly found in East Asia, but recent reports indicate that it is now also circulating in Europe (Hakze-van der Honing et al., 2011; Tesse et al., 2012). Like G3, G4 mainly causes sporadic cases of HEV infection (Inoue et al., 2006; Koizumi et al., 2004; Zhang et al., 2009). All four genotypes have a single serotype (Emerson et al., 2006a) which is an advantage in vaccine development as one vaccine potentially protects from all four genotypes. This is economically preferable in areas where more than one genotype is endemic as in for example China, where three of the genotypes are endemic (Wang et al., 1999) (More on vaccines in section 7.3.1.4).

7.2.3.1 Genotype 3

Genotype 3 is the main concern in Europe and America. The strains found in pigs and human are phylogenetically similar (Norder et al., 2009; van der Poel et al., 2001). In a phylogenetic study including human and porcine strains of HEV from a number of European countries the strains of

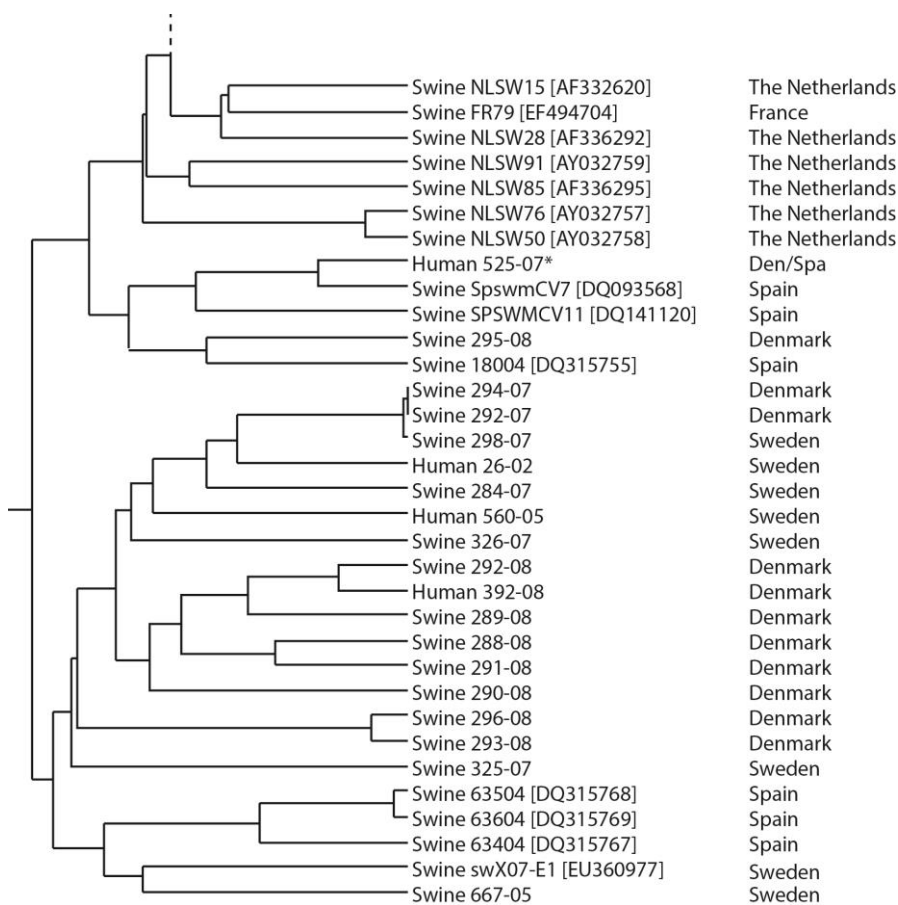


Figure 5 Phylogenetic relationship highlighting the geographic relatedness of HEV. The asterisk (*) marks the Danish person that had travelled to Spain

human and swine largely clustered together within countries. However, a Danish human strain was closer to the strains isolated from Spanish pigs (marked with * in Figure 5), when questioned the person had traveled to Spain (Norder et al., 2009). G3 has also been shown to be prevalent in many other animals such as: (roe-)deer (Reuter et al., 2009; Tei et al., 2003) wild boar (Matsuda et al., 2003). An animal that has been extensively investigated is rats as they are known carriers of other human pathogens, but also for the potential use as a small animal model. The first study focused on rats was in performed in Germany (Johne et al., 2010). The study was followed by an American study. In both cases G3 was not detected but a novel variant within the *Hepeviridae* family was discovered (See section 7.2.4) (Johne et al., 2010; Purcell et al., 2011). In a study by Lack et al. (2012) a high proportion of rats were found positive for G3 in especially American rats but also from other countries were also investigated. However, in an experimental infection of nude rats with G3, no signs of infection were observed and the infectivity of the inoculum was confirmed by infection in cynomolgus monkeys (Li et al., 2013). A strain closely related to G3 has later been found in rabbits (Cossaboom et al., 2011; Zhao et al., 2009). The rabbit strain has been shown to infect specific pathogen free (SPF) pigs as well as cynomolgus macaques (Cossaboom et al., 2012; Liu et al., 2013) and also belong to the same serotype (Wang et al.). Conversely, rabbits have been infected with G3 and G4, although infection efficiency of different strains varied. Rabbits, thus, have potential to function as a small animal model (Cheng et al., 2012). Wild game such as deer and wild boar has also been shown to be positive for G3 in both Japan and Europe (Kaci et al., 2008; Reuter et al., 2009; Sonoda et al., 2004). G3 have been shown to infect swine cells more efficiently than human cells. This is in accordance with the fact that pig herds worldwide are infected with HEV whereas human infections is sporadic and have not been reported to cause epidemics as it is often seen with G1 and G2.

7.2.3.2 Subtyping of HEV

Besides the division of HEV into genotypes which are approved by ICTV, research have been made to further sub-categorize the genotypes according to their genomic similarities. The different analyses were performed on full length sequences, but different or unspecified criteria were applied. In the study by Zhai et al. (2006), 37 complete genome sequences were analyzed and the four genotypes were further divided into 12 subtypes. In an analysis of 75 complete genome sequences the four genotypes and seven subclusters or subgroups were proposed (Okamoto, 2007). Most widely used is the subgrouping proposed by Lu et al. (2006) who studied 49 complete genomes and different subgenomic sequences. Here the four genotypes was recognized and these were divided into 24

subtypes (1a-e, 2a-b, 3a-j, and 4a-g). The criteria for allocation to the individual subgroups in G3 and G4 was a difference at nucleotide level of 12.1% – 18%. However, in recent studies that included novel full length sequences were not readily categorized by the method proposed by Lu et al. (2006) as they fulfilled the criteria of multiple subgroups (Bouquet et al., 2012; Oliveira-Filho et al., 2013). Recently it was proposed, that the subgroup divisions proposed so far was to be reconsidered and that a new set a rules regarding analytical criteria should be agreed upon (Smith et al., 2013).

7.2.4 Other animal specific HEV variants

In 1980, a virus was linked to the big liver and spleen disease in chickens observed in Australia and named big liver and spleen virus (BLSV). This virus was shown to be related to human HEV in 1999 (Payne et al., 1999). Two years later, a closely related strain, causing hepatitis-splenomegaly syndrome, was reported from Canada and USA. These North American strains were categorized as avian HEV (aHEV) (Haqshenas et al., 2001). Subsequent studies showed that aHEV was also widespread in Europe and Asia (Bilic et al., 2009). Recently, full length sequence analysis of BSLV and aHEV revealed that the genomes of the two viruses were approximately 6.6 kb long and 80 % homologous (Bilic et al., 2009). aHEV showed approximately 50 % nucleotide similarity with swine and human HEV variants (Huang et al., 2004). Though classified in the *Hepeviridae* family it has not been assigned to a genus. Based on the relatively low homology it has been proposed that a new genus should be made for aHEV (Meng, 2010; Meng, 2011). This notion is however dismissed by the phylogentic analysis performed by Smith et al. (2013). In recent years a number of species specific variants of HEV have been discovered in rat, bat, cutthroat trout, red fox and ferret (Batts et al., 2011; Bodewes et al., 2013; Drexler et al., 2012; Johne et al., 2010; Raj et al., 2012). Besides genotype 3 and 4 none of the variants or genotypes of HEV have yet been proven to have zoonotic potential, Avian HEV and rat HEV failed to experimentally infect rhesus monkeys (Huang et al., 2004; Purcell et al., 2011). Clinical disease has so far only been reported in humans and poultry (Hedrick et al., 1991; Purcell et al., 2011; Raj et al., 2012). In **Manuscript I**, the discovery of a HEV variant in farmed mink is described.

7.3 Epidemiology of HEV

7.3.1 Clinical disease in humans

Clinical disease in the population of the developed world is rare compared to the number of seroconverted individuals (Wedemeyer et al., 2012; Zhu et al., 2010). The clinical disease is generally comparable to hepatitis A virus (HAV) with an acute, self-limiting infection with non-existing/mild symptoms to fulminant hepatitis. The clinical signs associated with HEV infections are the same in developed and developing countries, indicating that genotype does not have an effect on clinical signs (Pavio and Mansuy, 2010). The most typical clinical signs are: Jaundice, fever, headache, nausea/vomiting, abdominal pain, anorexia, pruritus and hepatomegaly (Aggarwal, 2011; Dalton et al., 2008b; Pavio and Mansuy, 2010). The incubation period of HEV has reported to be anywhere from two weeks to two month (Purcell and Emerson, 2008). There have been two separate trials with volunteers letting themselves infect with HEV (Balayan et al., 1983; Chauhan et al., 1993). In both cases onset of symptoms appeared after one month e.g. anorexia, vomiting, abdominal pain and later followed by jaundice. Both cases also saw an elevation of alanine aminotransferase (ALT) and peak levels coincided with the most severe period of illness. ALT levels steadily returned to normal after peaking. Both subjects made full recovery and no indication of illness was remarked during the two year follow-up performed by Chauhan et al. (1993). Follow-up have also been performed on the experimentally infected person (Dr. Balayan himself). In the study by Balayan et al. (1983), here IgG

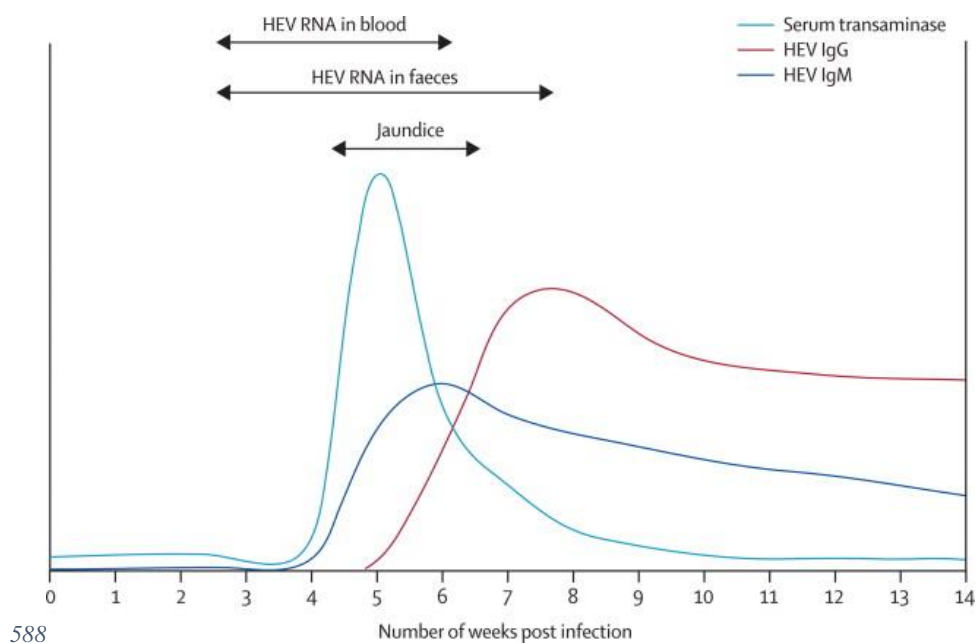


Figure 6 serological response to HEV infection and detection of HEV RNA in blood and feces (Dalton et al., 2008a). Early onset of viremia and fecal shedding is followed by a steep increase in Serum transaminase (ALT). This coincide with severe clinical signs such as jaundice. As ALT levels rises an IgM response is mounted followed by development of the persisting HEV IgG.

Table 1: Seroprevalences of the human population from several countries as well as data regarding genotype of the HEV found in humans animals and water. The data regarding genotypes of HEV is adapted from Kaba et al. (2013). The reference for the seroconversion data is noted.

Continent	Country	Sero-prevalence %	Reference (Seroprevalence)	Genotype of hepatitis E virus										
				1		2		3			4			
				H	W	H	W	H	A ^b	W	H	A ^c W		
Africa	Algeria	-		X										
	CAR	24	Pawlotsky et al. (1995)	X		X								
	Chad	-		X		X								
	DRC	-				X			X					
	Djibouti			X										
	Egypt	67.7	Fix et al. (2000)	X		X								
	Morocco	4	Bernal et al. (1995)	X										
	Namibia			X		X								
	Nigeria					X								
	Tunisia	22	Ben Halima et al. (1998)	X										
	South Africa	2	Grabow et al. (1994)		X				X					X
	Sudan			X										
Uganda			X					X						
Asia	Bangladesh	60.1	Sheikh et al. (2002)	X										
	Cambodia							X	X					
	China			X				X	X				X	X
	India	10-40	Acharya et al. (2006)	X	X								X	X
	Indonesia	0.5-20	Wibawa et al. (2004)										X	X
	Japan	4-19	Li et al. (2000)	X ^a				X	X				X	X
	Kyrgyzstan			X					X					
	Myanmar			X										
	Nepal			X										
	Pakistan	17.7	Hamid et al. (2002)	X										
	South Korea	17.7	Choi et al. (2003)						X	X				X
	Taiwan								X	X			X	X
	Thailand	8.6	Jutavijittum et al. (2000)						X	X				
	Uzbekistan			X										
Vietnam	9	Hau et al. (1999)	X										X	
Europe	Austria	11.4	Forgách et al. (2007)						X					
	Belgium	14	Van Hoecke et al. (2012)					X	X					X
	Denmark	20.6	Christensen et al. (2008)	X ^a				X	X					
	Czech Republic							X	X					
	Finland			X ^a										
	France	52.5	Mansuy et al. (2011)	X ^a				X	X	X				X
	Greece	0.23	Dalekos et al. (1998)					X						
	Germany	15.5	Krumbholz et al. (2012)					X	X					X
	Hungary	18.4	(Reuter et al., 2009)	X ^a				X	X					
	Italy	1.9	(Pavia et al., 1998)	X ^a	X			X	X	X				
	The Netherlands	2	(Bouwknegt et al., 2008a)					X	X	X				
	UK	16	(Dalton et al., 2008b)	X ^a				X	X	X				
	Russia			X				X						
	Spain	7.3	(Buti et al., 2006)	X ^a	X			X	X	X				
Sweden	9.3	(Olsen et al., 2006)	X ^a				X	X						
Switzerland	4.9	(Kaufmann et al., 2011)					X							
America	Argentina							X	X					
	Brazil	2.3	(Bortoliero et al., 2006)					X	X					
	Canada								X					
	Cuba	1.4	(Lemos et al., 2000)	X										
	Mexico	10.5	(Alvarez-Munoz et al., 1999)				X		X					
	USA	19-21	(Kuniholm et al., 2009)					X	X	X				
Uruguay							X							
Oceania	Australia								X					
	New Zealand	4	(Dalton et al., 2007)					X	X					

CAR = Central African Republic; DRC = Democratic Republic of the Congo.

^a Probable cases imported from an endemic area for genotype 1 HEV.

^b At least one positive sample from domestic pigs, wild boars, deer, mongoose or any combination.

^c At least one positive sample from domestic pigs, wild boars or any combination.

Ab's were still detected 12 years after inoculation (Balayan et al., 1994). These disease descriptions among experimentally infected volunteers closely resemble those reported in studies of epidemic and sporadic disease in areas where the disease is highly endemic. The course of HEV infection is summarized based on monitored UK cases of HEV infections in Figure 6 (Dalton et al., 2008a). Although clinical illness generally compare between different genotypes, the severity of the clinical signs can differ. Subjects infected with G4 rather than G3 tend to experience a more severe course of illness (Jeblaoui et al., 2013; Ohnishi et al., 2006). This manifests as higher rate of jaundice, elevation in ATL and bilirubinemia. Human infections with G3 have been shown to have a predilection for middle-aged to elderly males (Dalton et al., 2008b). The predominant infection of males is not observed with G4 infection and the subjects also tend to be younger (Jeblaoui et al., 2013). Studies in Swine have shown that infection with HEV is dose dependant (Meng et al., 1998b). The low number of clinical cases observed in humans, compared to the high prevalences reported in some regions (Table 1), may be due to low infectious dose that causes subclinical or fully compensated disease (Kuniholm et al., 2009; Lewis et al., 2010).

7.3.1.1 Chronic infections

Chronic infection with HEV has not yet been reported under normal circumstances. However chronic infections are linked to immunosuppressed individuals such as organ transplant recipients (heart lung liver and kidney) (Halac et al., 2012; Haagsma et al., 2008; Kamar et al., 2011; Kamar et al., 2010b; Pischke et al., 2012). Furthermore chronic HEV has been reported in human immunodeficiency virus (HIV) patients (Colson et al., 2009; Dalton et al., 2009). Chronic infections led to consistently elevated ALT values and prolonged excretion of HEV. Although illness in chronically infected individuals can be severe leading to cirrhosis, some, like the general population, never experience any sign of HEV infection (Kamar et al., 2011). Chronic infections have successfully been treated by different approaches such as; reduction of immunosuppressant medication (Kamar et al., 2010a), administration pegylated interferon alfa (Haagsma et al., 2010) and use of the antiviral drug ribavirin (Mallet et al., 2010).

7.3.1.2 Neurological manifestation

Several reports confirm HEV infections in patients with Guillian-Barré syndrome (GBS) (Kamani et al., 2005; Loly et al., 2009; Maurissen et al., 2012). The illness presents as loss of strength in arms and legs and later paralysis. GBS induced by viral hepatitis is well described in the literature with previous reports attributed to infection by HAV and hepatitis B virus (HBV) (Tabor, 1987). The syndrome caused by HEV has been observed both in Europe and India, but the genotype have not

been resolved. However, the geographic distribution indicates that both G1 and G3 could be implicated as European subjects had no travel history (Maurissen et al., 2012).

7.3.1.3 *HEV in pregnancy*

HEV has been reported to have a mortality rate of more than 20 % in pregnant women (Krawczynski, 1993; Kumar et al., 2004). The studies supporting these numbers are based on infections with genotype 1 and 2. Thus, the mortality rate of pregnant individuals infected with HEV G3 and G4 is unknown (Mesquita et al., 2013). Vertical transmission of HEV has also been demonstrated in six of eight children born to women with hepatitis E infections (Khuroo et al., 1995). Since, it has been discovered that pregnant women with acute liver failure have a higher viral load than non-pregnant women or men with similar symptoms during HEV infection (Borkakoti et al., 2013).

7.3.1.4 *Vaccination*

Vaccination would be especially advantageous in developing areas where seasonal epidemics of enteric disease are common because of low sanitary conditions and rudimentary sewage systems. There are two candidate vaccines in development. Both are based on recombinant technology and on G1. One vaccine is developed by GlaxoSmithKline and consists of a 56 kDa recombinant protein from ORF2. In a phase 2 study the vaccine appeared immunogenic and well tolerated with a protection of 95.5% against HEV (Shrestha et al., 2007). An already released vaccine, HEV 239, was developed by a Chinese biotech collaboration. It is currently only available in China but negotiations with the World Health Organization (WHO) are ongoing (Park, 2012). The primary component of the vaccine is a 26 kDa protein translated in *E. coli* from ORF2 which produces 23 nm VLP. The vaccine has gone through Phase 3 clinical trials where approximately 48,000 people were administered three doses of the vaccine. The negative control group of comparable size was administered hepatitis b virus vaccine. 100% protection was observed against HEV and the vaccine caused no or very mild side-effects (Zhu et al., 2010). Pregnant women were inadvertently also vaccinated during the trials and showed no adverse effect of the vaccination (Wu et al., 2012). Pregnant women in the developing world where G1 and G2 are endemic would benefit greatly from a HEV vaccine due to the greater implications for this group of individuals. However, HEV epidemics are most prevalent in the poorest part of the world and recent outbreaks have occurred in refugee camps in the continent of Africa (Ahmed et al., 2013; Teshale et al., 2010b; The UN Refugee Agency, 2012) which means that the vaccination might not be a possibility for the people who need it the most for economic reasons (Zhang et al., 2013).

7.3.2 Clinical disease in pigs

HEV G3 is present worldwide in pigs and the herd prevalence is high (Table 2). Pigs do not show symptoms of clinical illness during infection with HEV, although when necropsied mild lesions of the liver of infected animals were reported (Halbur et al., 2001; Meng et al., 1997). However in the first report of experimental infection of pigs with an isolate from humans, that later was revealed to be of swine origin (Lu et al., 2004), clinical signs similar to those reported in humans were reported (Balayan et al., 1990). Most studies on liver lesions have been performed on pigs experimentally infected with HEV, but has also been reported in a study clarifying if there is a correlation of PMWS and HEV infection in naturally infected pigs (Martin et al., 2007). There was a positive correlation between lesions of the liver and HEV, however PMWS and HEV was not directly associated (Martin et al., 2007). In naturally infected pigs with no other illnesses, hepatic lesions has been reported to be mild or non-existing. Interestingly, in a study of naturally infected pigs, lesions of the liver did not correlate with the detection of HEV in the liver (de Deus et al., 2007).

In experimentally infected pigs, fecal shedding of virus is often the first sign of HEV infections (Feagins et al., 2008a; Meng et al., 1998a) and are detectable within a week of inoculation (Feagins et al., 2008a; Halbur et al., 2001), the response does however seem to be dose dependent (Meng et al., 1998a). The fecal shedding is followed by viremia which again is followed by IgM Ab development. IgG Ab's are developed immediately after and persists (Meng et al., 1998a). Experimental inoculation via the oral route have proved difficult (Bouwknegt et al., 2008b;

Table 2 Seroprevalences of the HEV Ab's of domestic pigs in different countries

Country	Number of			References
	tested	positive	%	
Argentina	97	22	22.7	Munne et al. (2006)
Brazil	357	227	63.6	Vitral et al. (2005)
Canada	712	129	18.1	Meng et al. (1999)
Canada	998	594	59.5	Yoo et al. (2001)
China	82	22	25.0	Meng et al. (1999)
China	419	330	78.8	Wang et al. (2002)
Denmark	213	156	73.2	Breum et al. (2010)
Germany	1072	534	49.8	Baechlein et al. (2010)
Great Britain	256	219	85.5	Banks et al. (2004)
India	284	122	43.0	Arankalle et al. (2002)
Indonesia	99	71	72.0	Wibawa et al. (2004)
Korea	140	57	40.7	Meng et al. (1999)
Mexico	125	8	6.0	Cooper et al. (2005)
New Zealand	72	54	75.0	Garkavenko et al. (2001)
Spain	60	15	25.0	Pina et al. (2000)
Sweden	204	118	58.0	Banks et al. (2004)
Taiwan	275	102	37.1	Hsieh et al. (1999)
Thailand	76	10	13.0	Cooper et al. (2005)
Thailand	75	23	30.7	Meng et al. (1999)
The Netherlands	34	8	23.5	Banks et al. (2004)
USA	293	202	68.9	Meng et al. (1999)
USA	84	29	34.5	Withers et al. (2002)

Kasorndorkbua et al., 2004). However, pigs are readily infected intravenously by G3 and G4 HEV (Meng et al., 1998b). Pregnant gilts experimentally infected with HEV did not transmit HEV to their fetus or newborn even when shedding HEV fecally (Kasorndorkbua et al., 2003).

In naturally infected pigs viremia is reported to be transient or not detected and viral loads are often inferior to those in fecal samples (Kanai et al., 2010). Like viremia, fecal shedding is not always detected. Maternal antibodies (MatAb's) are successfully transferred to the piglets if the Ab level of the sow is sufficiently high (Casas et al., 2011; de Deus et al., 2008; Meng et al., 1997). The role of MatAb's are however unclear (Kanai et al., 2010). Onset of HEV infection in swine herds are reported to occur at 7 -13 weeks of age based on shedding and Ab development (Casas et al., 2011; de Deus et al., 2008). Using in-situ hybridization, HEV has proven to be present extra hepatic in large intestine, small intestine, bile duct, spleen, lymph nodes, tonsils, and kidney (Choi and Chae, 2003) and many of these findings were confirmed in another study by RT-PCR (de Deus et al., 2007). In both studies, no muscle samples were tested. Detection of HEV in a large fraction of muscles samples from pigs contact infected with swine HEV has was described by Bouwknegt et al. (2008a) posing a threat to pork meat consumers if infection of HEV occurs late in the fattening period.

HEV infection dynamic is the focus of **Manuscript IV** (section 0) of this thesis, where the effect of MatAb's as well as the presence of HEV in pigs at slaughter was investigated in a large scale longitudinal study.

7.3.3 Routes of HEV transmission

The possible routes of transmission of HEV are plentiful, some of them are well described, but some still lack substantial evidence. In Figure 7 the plausible routes of transmission have been illustrated.

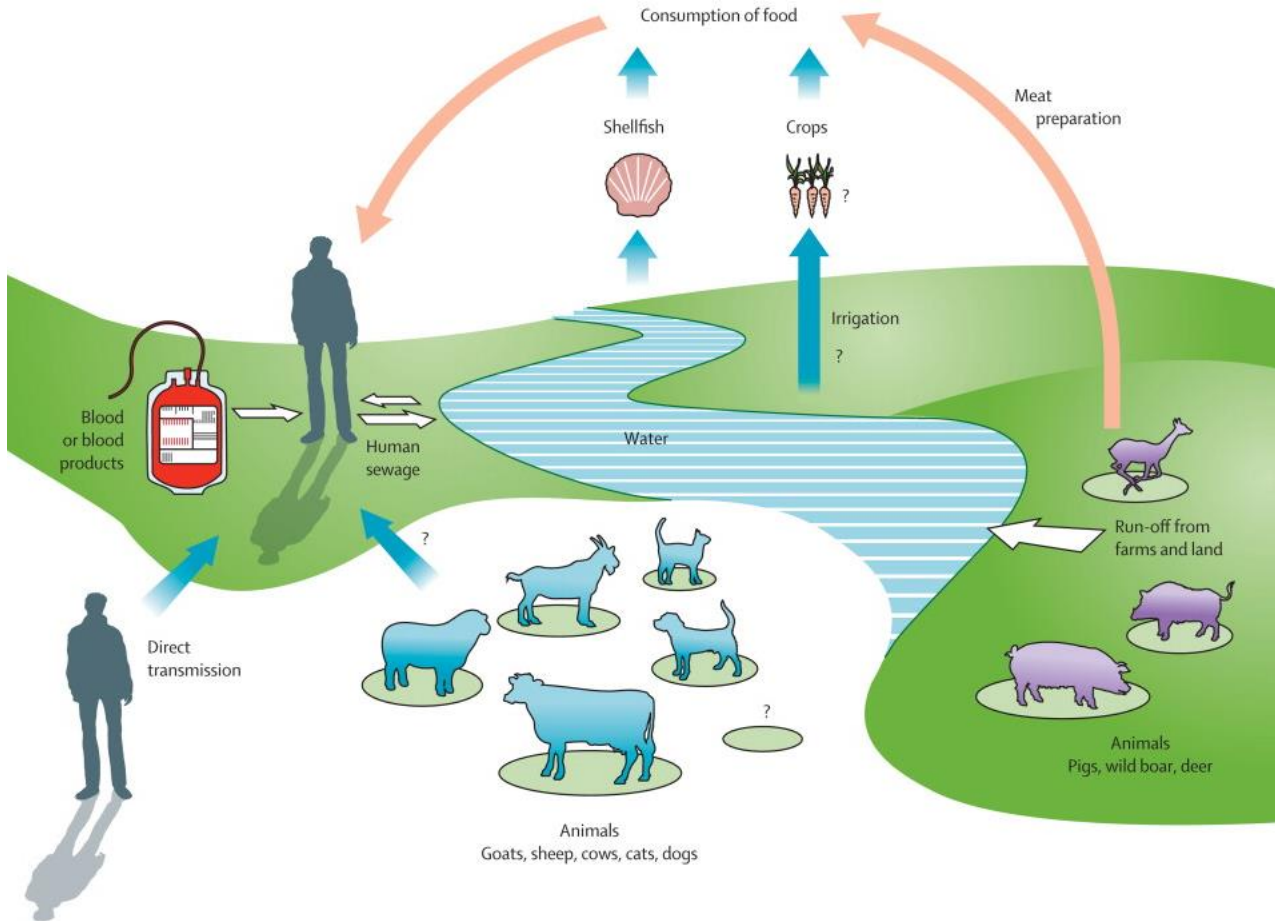


Figure 7: Possible and confirmed routes of transmission of HEV from reservoirs to humans. Taken from Kamar et al. (2012).

7.3.3.1 Waterborne Transmission

Waterborne transmission is the most important route of transmission in developing countries (Guthmann et al., 2006; Hazam et al., 2010; Howard et al., 2010; Wong et al., 1980). The epidemics are often caused by fecal contamination of water supply typically by waste water spill over. Also the environmental water reservoirs such as rivers has proven to be contaminated by HEV. In the study by Verma and Arankalle (2010) an increased number of samples were found positive from the Mutha river compared to previous years. The contaminated river water poses an elevated threat of drinking water contamination during monsoon because of river overflow. The genotypes associated with

waterborne epidemics is G1 in Asia and Africa and G2 in Africa and Mexico (Teshale et al., 2010c). Environmental factors are further discussed in section 7.4 and in **Manuscript II**.

7.3.3.2 Direct transmission

The direct transmission from human to human or even animal to human is not well described. The higher seroconversion of farmers and swine veterinarians compared to people with no contact to live pigs support that a direct transmission might occur (Christensen et al., 2008; Drobeniuc et al., 2001; Lee et al., 2013). Also a butcher in England and a slaughterhouse worker in Spain have been diagnosed with acute HEV infection (Jary, 2005; Pérez-Gracia et al., 2007).

Another study showed that intra familial transmission was negligible even during epidemics (Aggarwal and Naik, 1994). It has even been suggested that humans generally are a poor vector for the zoonotic variants of HEV (Li et al., 2006). However, in 2008 during an epidemic in Sudan a second wave of infections resulted in a prolonged epidemic. As water sanitation programs were instated, the source of infection was removed. Therefore it was speculated that the prolonged duration of the epidemic was due to person-to-person infections (Teshale et al., 2010a).

In epidemiological studies, owning pets were correlated well with seroconversion of their owner (Banks et al., 2004; Kuniholm et al., 2009; Widdowson et al., 2003). In a specific case, the pet cat, of a Japanese patient that had contracted HEV infection, was positive for anti-HEV Ab's (Kuno et al., 2003). In another study 33% of cats was shown to have anti-HEV Ab's (Okamoto et al., 2004). However, no study has definitively linked HEV transmission to pets. Rats have also been shown to carry G3 HEV (see section 7.2.3.1) and are therefore also a possible source. The level of anti-HEV Ab's in American rats have been shown to be between 4.5 % and 90 % depending on the geographic location (Favorov et al., 2000; Kabrane-Lazizi et al., 1999a).

7.3.3.3 Vertical transmission (Parenteral Transmission)

HEV infected pregnant women are at special risk to develop complications (more in 7.3.1.3). There is strong evidence that the offspring, if it survives, can develop fulminant hepatitis shortly after birth (Khuroo et al., 1995; Khuroo and Kamili, 2009). Breastfeeding was not identified as a risk of transmission of HEV between mother and child (Chibber et al., 2004).

7.3.3.4 Blood transfusion and Organ transplants

There are well documented cases of transmission of HEV by blood transfusion where matching genomic sequences have been recovered from donor and recipient (Boxall et al., 2006; Colson et al.,

2007; Matsubayashi et al., 2008). In a study from UK, 880 minipools corresponding to 42,000 individual donors were tested for the presence of HEV RNA and 6 (0.7 %) were found positive and the samples positive were also positive for IgG. It is estimated that 75 % of donated blood is given to immunosuppressed patients in the UK, who have previously been shown to be at risk of developing chronic infections (Bihl and Negro, 2009) (see section 7.3.1.1 for more on chronic infections). The low prevalence of RNA HEV was highlighted by a recent study from USA where 1939 individual blood donors were tested but none were positive for HEV RNA even though 16 % tested positive for anti-HEV IgG (Xu et al., 2013)

7.3.3.5 *Foodborne zoonotic transmission*

There is a limited number of well documented zoonotic incidents related to ingestion of infected food, where the strain of HEV retrieved from the patient has been subsequently been found in the food. However there are more reports of HEV being present in the food supply chain (Berto et al., 2012; Di Bartolo et al., 2011; Leblanc et al., 2010). The difficulties to identify the source of HEV arise from the long incubation period from ingestion of food until onset of clinical symptoms. In this period of two weeks or more, leftover food has typically been disposed. But in rare cases it has been frozen (Tei et al., 2003). The well documented cases have been instances with low incubation period and severe disease. A popular example of foodborne transmission comes from the south of France where the liver sausage, Figatelli, is traditionally consumed raw. This led to the development of hepatitis only in the three family members of five that consumed the sausage (Colson et al., 2010). Virus isolated from French pig liver sausages has also proven to be infectious in 3D cell culture systems (Berto et al., 2013a) (cell cultures are further described in 7.5.1).

In Japan, two men independently were admitted to a hospital with severe hepatitis. It was later discovered that the two men were acquainted and together had eaten the uncooked liver of a boar (Matsuda et al., 2003). Many studies have proven that HEV is prevalent in livers bought at grocery stores in different countries; Netherlands (4 %), France (4 %), Southern Germany (4 %), India (0.8 %) and Japan (2 %) (Bouwknegt et al., 2007; Kulkarni and Arankalle, 2008; Rose et al., 2011; Wenzel et al., 2011; Yazaki et al., 2003). However, a much larger fraction of livers was found positive in USA (11 %) (Feagins et al., 2007). Feagins et al. (2007) also showed that the virus isolated from these livers were infectious in some cases. Thus, consumers of pork livers are exposed to HEV. In multiple studies there was a positive correlation between the consumption of liver or other types of organs (Kuniholm et al., 2009). Frozen berries have previously been associated with outbreaks of gastroenteritis and found positive for HAV and NoV (Le Guyader et al., 2004; Niu et al., 1992; Nordic

outbreak investigation team, 2013). The contamination of berries is speculated to arise from low sanitary conditions at the production site, but also the quality of water used for irrigation has been questioned. In a study investigating the effect of using river water for irrigating a plot of strawberries showed that HEV was present in a sample of strawberries, but not in the water samples used for irrigation (Brassard et al., 2012).

In **Manuscript IV** the prevalence of HEV in livers bought in grocery stores, as well as a study on pigs at time of slaughter highlight the presence of HEV at consumer level.

7.3.4 Inactivation of HEV

Inactivation of HEV in food products are not thoroughly investigated. General practices regarding diminishing the viral load in food stuff includes treatment with chloride, chlorine dioxide and ozone. These practices, however, have varying effect on different viruses and the effect on HEV has not been evaluated. Thermal treatment of food is widely used, and often also applied by the consumer (Hirneisen et al., 2010). Therefore it is important to set guidelines for the heat treatment to dispose of HEV. It was recommended by the US government that pork is heated to min. 71°C, this has however recently been lowered to 62.8°C but added an additional 3 minutes rest time (USDA, 2011). Emerson et al. (2005) assayed the stability by heating fecal suspensions containing HEV to 45 – 70°C and inoculate cell systems with the heat treated suspension. Three different strains of HEV was assayed and were all infectious but greatly reduced in infectivity, after heat treatment at 60°C for one hour. In the study, HEV was not in the protective environment of meat and furthermore the strains had been frozen and thawed previously, maybe having a negative impact on stability. HEV was also shown to survive heating to 56°C in the in vitro experiment (Emerson et al., 2005). These findings was supported in another study where liver homogenates heated to 56 °C for one hour were inoculated intravenously into pigs and resulted in 4 out of 5 pigs developed a HEV infection (Feagins et al., 2008a). When testing for infectivity of HEV in small cubes of intact pork liver, that had been stir fried or boiled to a center temperature of 71°C, no signs of HEV infection was observed when inoculated into pigs (Feagins et al., 2008a). In a third study, HEV infected liver was used to make a pâté like substance to mimic processed foods where fat, spices and salts where added. The substance was heated to 62 68 or 71°C for 5 10 and 20 min. and inoculated into pigs. The only preparation that did not result in infection of the pigs were the one heated to 71°C for 20 minutes. This further showed that preparation of HEV containing food products requires thorough heating to a minimum temperature of 71°C (Barnaud et al., 2012).

7.4 Environmental

Viral contamination of water reservoirs can happen in a number of ways. Contamination by human pathogens are often due to failure in septic systems and wastewater treatment systems (Borchardt et al., 2011). Furthermore, extreme weather with heavy rain can lead to failure of the sewage system and lead to the direct contact between human and contaminated waste water by contaminating lakes and beaches used recreationally (reviewed in (Sinclair et al., 2009)), flooding of basements and houses as seen during the flood in Copenhagen in 2011 (Wójcik et al., 2012) and by entering groundwater wells (Kukkula et al., 1997). Viruses accounted for 23 % of acute gastrointestinal illnesses caused by drinking water of groundwater origin in the US (Brunkard et al., 2011). The human viruses often associated with contamination of drinking water is adenovirus, enterovirus, HAV and NoV (Borchardt et al., 2012). In Denmark, 99% of all drinking water originates from groundwater that includes the municipal and private wells. The groundwater is aerated and sand filtered but no further treatment is performed (Spliid and Køppen, 1998). Water quality in municipal groundwater wells is monitored for the presence of coliforms, but viral analysis is not performed routinely. The lack of viral control in water is a world-wide concern and has been addressed by the WHO and Organization for Economic Co-operation and Development (OECD) stating that from a technological and more importantly economic standpoint, implementation of virus monitoring is still not feasible (Dufour et al., 2003; OECD, 1998). From a zoonotic point of view the large and increasing production of animal waste is a concern as it is spread on a vast area often in contact with water reservoirs or has the potential to leach into the groundwater through the soil. In Denmark and the United Kingdom; 26 and 90 million ton of livestock waste is spread on fields each year, respectively (Knowledge Centre for Agriculture, 2012; Smith et al., 2001). The length of transportation for some viruses have been shown to be > 400 m horizontally and >60 m vertically (Keswick and Gerba, 1980). Therefore the placement of groundwater drillings should be considered with respect to agricultural activities.

7.4.1 HEV in the developed world and stability

There is a limited and conflicting knowledge on the stability of HEV. HEV has been described as being stable in the environment, but labile in a laboratory setting, in that freeze-thawing cycles were particularly damaging to the particle (Balayan, 1997). The environmental stability of HEV and HAV was compared in a study, by spiking soil with HEV and HAV from infected humans. The samples were left at fluctuating environmental temperature (ET) and at 37 °C. Survival at ET was very similar between the two viruses with HEV surviving 9 weeks and HAV 8 weeks. The detection of virus was performed by real time RT-PCR and thus does not reflect actual infectious particles. At 37 °C HAV

was more stable than HEV with 13 and 10 weeks respectively (Parashar et al., 2011). Soil composition and hydrology of the soil have a huge impact on virus survival in soil, therefore, this study only represent one scenario. Different survival times could be possible under other circumstances (Hurst et al., 1980). Survival in water is an important factor in epidemics. The stability of HEV was tested in sewage water, where HEV G1 was spiked in both phosphate-buffered saline solution (PBS) and sewage and incubated at 20 °C for two month. After one month HEV had fallen to 1% in sewage and after two was not detected. In PBS the titer was unchanged after two months (Pina et al., 1998). The presence of HEV in sewage and wastewater has been shown in multiple studies. Not surprisingly, wastewater from slaughterhouses have been found positive for HEV in Spain and all positive samples belonged to G3 (Clemente-Casares et al., 2009; Pina et al., 2000) and urban wastewater from France and USA have tested positive for HEV (Clemente-Casares et al., 2003). Furthermore urban sewage from Barcelona have been extensively investigated on two accounts, showing a persistent presence of HEV G3 in 92 % of typed samples but surprisingly also G1 in the remaining 8 % (Clemente-Casares et al., 2003). In Switzerland a survey of inflow and effluent of multiple wastewater treatment plants (WWTP) in the Canton of Zürich showed, that 32 % of influent samples were positive for HEV, however, no effluent samples were positive. The detection limit of the PCR assay used for detection was already reached in the influent samples and as analysis of human adenovirus (HAdV) in the same samples showed a reduction from influent to effluent it is highly likely that HEV is present in effluent but in lower concentration than detectable by the assay (Masclaux et al., 2013). The notion that sensitivity of the assay and not complete removal of HEV was the case, was supported in a study by Jothikumar et al. (1993) that detected HEV in both inlet and effluent samples in WWTP in India. In Italy, HEV was similarly detected in 16 % of inflow samples from WWTP, interestingly, G1 sequences was detected in all but one of the 19 positive samples (La Rosa et al., 2010). That HEV entering the WWTP is infectious was made probable by comparing WWTP workers to a group of same socioeconomical status but working in an office. The seroconversion of WWTP workers was 50 % compared to 30 % in the group working in an office (Albatany and El-Shafie, 2011). In these urban waste water studies, mainly humans are the source of HEV potentially released into the water environment. Animals and especially pigs are also a plausible contributor of contamination with HEV to the water environment. Animal waste is stored in slurry pits until distribution on land plots. In a study performed on slurry pits in America the nearby water reservoirs was sampled for presence of HEV, but none were positive. The pig slurry was, however, positive for HEV that proved infectious to naïve pigs (Kasorndorkbua et al., 2005). The water samples included in the study was not taken

after application of slurry to land. The result therefore only indicates that spill over from the slurry pits to water environment does not happen. In a study performed on American households, having a private well for the main supply of tap water was negatively correlated with the detection of anti-HEV Ab's (Kuniholm et al., 2009). Contrary, in a national study in France consumption of well water correlated positively to HEV infection and so did consumption of shellfish (Renou et al., 2008). In **Manuscript II**, leaching of HEV, rotavirus, porcine circovirus type 2, somatic coliphages, *Enterococcus* spp. and *E. Coli*, naturally present in pig slurry, through soil into tile drains of under field conditions is studied to elucidate if contamination from pig slurry to the water environment occurs.

7.4.2 Shellfish

The quality control in shellfish production solely relies on bacterial testing and no viral control are implemented. This is in full compliance with European and American legislation (Anonymous, 1991; FDA, 2009). In Europe, the limit of microbial contamination is based on number of coliforms in 100 g of shellfish meat whereas the US legislation relies on *E. coli* in water samples taken from the shellfish beds. If bacteria are detected then the shellfish should be treated before being sold. The treatment is called depuration and is performed by moving the shellfish to pure water and let them purge bacteria and virus naturally. The problem with this method is that shellfish efficiently purge bacteria but not virus (Loisy et al., 2005; Love et al., 2010; Schwab et al., 1998). Consumption of shellfish have been the cause of large epidemics, one of the largest was in 1989 where 290.000 cases of HAV infections were reported in Shanghai that were related to the consumption of clams (Tang et al., 1991). As consumption of seafood is inclining so are the reports of seafood associated infections from only 3 reports in the 1970s to 22 reports in the 1990s (Potasman et al., 2002). Sporadic cases of HEV have also been linked to consumption of shellfish and the detection of HEV in coastal shellfish have also been reported (Crossan et al., 2012; Song et al., 2010), in one case with prevalences above 90% from mussels collected near a slaughterhouse. In Italy, shellfish was used as a bio-marker for marine contamination. Pure mussels are placed in an area suspected of contamination and after a period of time tested for pathogens. In the study HEV was detected in 8% of the mussels all placed in area not suitable for harvesting of shellfish. Interestingly, the HEV found in these shellfish was G1 not considered endemic in Europe.

The presence of HEV in blue mussels produced in Denmark was investigated along with PCV2 and Rotavirus in **Manuscript III**.

7.5 Detection and diagnostics

7.5.1 Cell culture

In general, the use of cell cultures for detection of viruses for diagnostic purposes is very limited as newer and faster methods of detection (real time PCR) has replaced cell cultures. Cell cultures are, however, still the preferred methods for test of infectivity of a given sample but also inoculation into susceptible small laboratory animals can be used for infectivity studies (Leland and Ginocchio, 2007). The lack of an efficient cell culture system and small animal model for HEV has hampered the research of the molecular characterization, infection and egression of the viral particles from cells. Furthermore the infectivity of food products that have tested positive for HEV by real time PCR have not easily been tested. For assessment of the infectivity, and potential threat of public health of a given foodstuff, pigs have been used as infection model. This is by no means an optimal detection method for numerous reasons such as price, labor, throughput and ethical reasons. Growing HEV in cell culture has proven very difficult. It is still not an easy endeavor to undertake. A cell line that accepts any strain from any material is still not identified. There are numerous reports of cell culturing systems supporting growth of adapted strains of HEV.

The first efficient cell culturing system of HEV was reported by Tanaka et al. (2007) who recommended the PLC/PRF/5 cell line. This cell line was identified after testing 21 different HEV strains. PLC/PRF/5 proved most effective though the cell line A549 also showed potential. This procedure has since been tried reproduced by other laboratories, but have so far not been successful (Berto et al., 2013b). The speculation was that the success of the initial trial was connected to the specific strain used, in this case a highly positive human G3 fecal sample (JE03-1760F). Since HEV is an RNA virus the strain is in reality a myriad of different virus “variants” termed quasispecies (Grandadam et al., 2004). Some of these quasispecies may have the ability to efficiently infect only certain cells (Shukla et al., 2011). This could also explain the high viral load needed to infect cells, as only a fraction of the virus inoculated are in fact infectious to that particular cell. Later the method was expanded to also support the propagation of the G4 strain HE-JF5/15F (Tanaka et al., 2009). In this development, the cell line A549 played a crucial role as it supported the propagation of strains already adapted in PLC/PRF/5, more efficiently. The propagation of HEV isolated from serum has also been demonstrated using this system. Here the propagation of a wide variety of strains from G1, G3 and G4 was successful only if the inoculum had a titer of more than 3.5×10^4 particles (Takahashi et al., 2010). Although the cell culture system appeared efficient there are still some problems. The PLC/PRF/5 cell line has hepatitis B virus genes incorporated into its genome, which can have an

effect on the processes of HEV infection. Furthermore, the cell line A549 is not a hepatic cell-line therefore propagation in this cell line may not mimic the replication in livers (Rogée et al., 2013). A consequence of this might be the association of lipids and ORF3 protein observed in the cultured virions using the PLC/PRF/5 and A549 system and they were not precipitated by neutralizing antibodies directed against ORF2. However, this association of ORF3 and lipids were also seen in the virions in the serum samples used for inoculation that also proved to be able to infect the cell lines in the presence of antisera. This phenomenon is not described for virions excreted in feces. It was argued that the virions produced were shed of the lipid layer and ORF3 in its passing through the bile duct when encountering detergents in the digestive system (Takahashi et al., 2010).

A recent study suggested that HEV has a high possibility of crossing many species barriers as it is already seen with G3 (Shukla et al., 2011). Cell lines from 10 different species were inoculated with the Kernow-C3 Genotype 3 strain, which was isolated from a HIV patient with a persistent infection (Dalton et al., 2009). Kernow-C3 was able to infect cell lines from all 10 different species although inefficiently in most cases. This was attributed to the high titer and a set of broadly infectious quasispecies during the persistent infection of the immunocompromised HIV patient (Shukla et al., 2011). The human cell lines used included PLC/PRF/5, A549 and HepG2/C3A. The latter proved to support HEV propagation most efficiency (Shukla et al., 2011). The viral progeny produced in the HepG2/C3A was not all infectious and many was carrying the ORF3 protein as well as lipids as observed by Takahashi et al. In the work of Shukla et al. (2011) it was however, described as an abnormality of the cell culturing process, even hypothesizing that a useful cell culture would not be possible.

Berto et al. (2013b) also utilized the PLC/PRF/5 cell line, but followed another strategy of culturing HEV. Here a 3D system was applied, that had previously proved efficient in the attempts to culture NoV (Straub et al., 2007). The use of 3D cell lines have proven to give a more natural tissue-like distribution of receptors on the cells, which more closely mimicking the *in vivo* conditions. The produced viral particles were infectious and retained a proper size and morphology (Berto et al., 2013b). The strain used was isolated from the liver of an experimentally infected pig in the study by Bouwknegt et al. (2008b).

In a very recent publication the aim was to validate a human and porcine cell line that exhibited natural hepatic functions throughout infection, which is not the case using PLC/PRF/5 with the HBV genes incorporated or the lung cell line A549. The cell lines chosen were the human HepaRG and

porcine PICM-19 stem-cell cell line capable of proliferating into hepatic cells. The outcome was two cell lines that were able to support a full cycle of HEV infection, however, the titers of progeny virions observed were low compared to titers obtained using the PLC/PRF/5 and A549 combination (10^3 vs 10^7 , respectively) (Rogée et al., 2013). The system of having a porcine and human cell line bodes well for the study of the zoonotic potential of different strains.

7.5.2 Real time PCR

Real time PCR is presently the golden standard for the detection of viruses in diagnostic laboratories. The reason is high specificity, sensitivity and throughput. There are different chemistries that allow for monitoring the PCR process in real time. All are based on excitation of molecules and recording the intensities of emission (Kubista et al., 2006).

7.5.2.1 SYBR green

SYBR green is a reporter dye that binds to dsDNA and the complex SYBR green-dsDNA complex is excitable by blue light and emits green light. As the amount of double stranded DNA increases exponentially during the PCR amplification process, so does the signal intensity of the increasing amount of SYBR green-dsDNA complex (Kubista et al., 2006). The assay also allows for melt analysis which can confirm that the product amplified is the expected. This is done by lowering to below annealing temperature and then incrementally increase temperature with recording of signal at each increment. A drop of signal will occur as the SYBR green-dsDNA complex is disrupted due to melting of the dsDNA (Ririe et al., 1997). An early real time assay directed against HEV was based on SYBR green (Oru et al., 2004) It was however not validated against circulating genotypes of HEV and used short primers potentially posing a specificity issue (Jothikumar et al., 2006).

7.5.2.2 Primer Probe Energy Transfer (PriProET)

PriProET uses a primer probe system but lends its chemistry from fluorescence resonance energy transfer (FRET) systems (Rasmussen et al., 2003). The principle of FRET is to transfer energy from the donor fluorophore to an acceptor fluorophore (reporter). A fluorophore is a molecule that is able to be excited by light at a given wavelength and after excitation emit light at another wavelength. In FRET, the acceptor is required to be excited at the wavelength emitted by the donor. A commonly used fluorophore pair is FAM (Donor) and Cy5 (acceptor). FAM has an energy emission profile that overlaps the excitation profile of Cy5, thus, if FAM and Cy5 are in close proximity excitation of FAM will lead to excitation of Cy5. This is utilized in PriProET by labelling the reverse or forward primer with a donor fluorophore (e.g. FAM), and the probe with an acceptor fluorophore (e.g. Cy5).

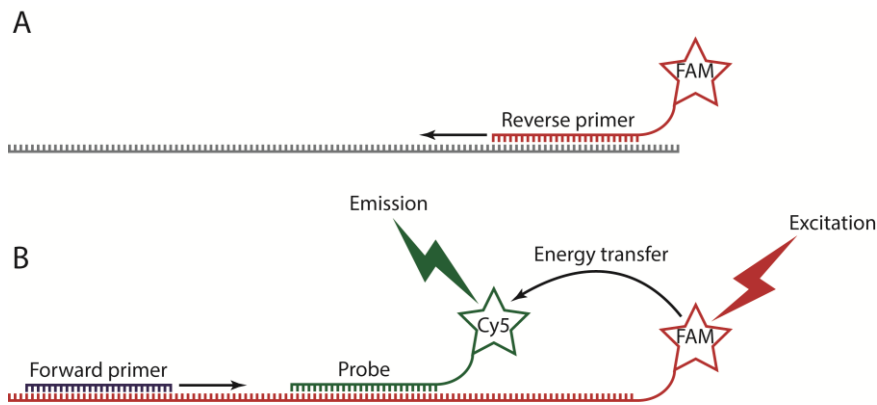


Figure 8: A Annealing of the labelled reverse primer resulting in a labelled cDNA. B Annealing of the forward primer and probe, excitation is performed during the annealing step, as the probe is removed during elongation. Excitation of the incorporated FAM molecule leads to energy transfer to the Cy5 labelled probe. This in turn emits measurable light which is recorded by the real time PCR cyclers.

The labelled reverse primer is incorporated into the cDNA of the template (Figure 8A). When the probe has annealed to the cDNA (Figure 8B) the PCR cyclers excite the donor fluorophore (FAM) at an appropriate wavelength and energy transfer to the Cy5 labelled probe occurs. This energy transfer excites the donor and allows for emission of light at a different wavelength which is recorded by the PCR cyclers. The method has some advantages. The primer probe system and the requirement of the probe to be located close to the incorporated donor fluorophore increases the sensitivity of the assay thus minimizing false positives. The probe has tolerance for some mismatches, an advantage when detecting RNA viruses as the mutational rate is high (Rasmussen et al., 2003). Another feature available is the possibility to perform melt analysis. The analysis is performed similar to SYBR green assays, but the melting point is directly referring to that of the probe. Mismatches in the probe will reduce melting point compared to perfectly matched probes. If designed correctly this feature can be

utilized to differentiate genotypes. This is the case in the PriProET assay developed for detection of HEV by Breum et al. (2010). Here the probe has a perfect match with genotype 3 and 4 and a double mismatch with genotype 1 and 2. This allows for immediate differentiation between zoonotic- and exclusively human genotypes when screening human samples, and also provide a mean to validate positive results (Breum et al., 2010).

7.5.2.3 TaqMan

Taqman chemistry requires a set of primers and a dual labelled probe much like PriProET. However the probe is dual labelled with a fluorophore and a quencher residing on each end of the probe. As long as the probe is intact no signal will be given from the fluorophore as the quencher will absorb the energy when excited by the real time PCR cyclers. When the probe is correctly annealed downstream of the primer, it will be degraded at the elongation step of the PCR cycle by the DNA polymerase (with 3' exonuclease activity), resulting in a detectable signal as the fluorophore is released from the quencher (Kubista et al., 2006).

The Taqman assay developed by Jothikumar et al. (2006) is directed against the same region as the assay developed by Breum et al., (2010) and is widely used with high sensitivity. It does, however, not feature the genotype discrimination feature, as melt analysis is not possible using Taqman because of probe degradation. Another Taqman assay developed for detection of HEV is located in a less conserved region but still within ORF2. As the Taqman and PriProET chemistries are interchangeable if the probe and primers are positioned correctly, the assay was evaluated using both chemistries. The TaqMan assay performed better overall, with higher sensitivity and efficiency (Gyarmati et al., 2007). However, the PriProET assay did feature genotype discrimination.

7.5.3 ELISA

The detection of antibodies using Enzyme-linked immunosorbent assay (ELISA) is an important tool to estimate the epidemiology of HEV. The assays used to detect HEV are largely based on ORF2 capsid proteins, but ORF3 are added for extra sensitivity, however ORF3 directed Ab's is suggested to have a shorter half-life (Ghabrah et al., 1998), thus assays using these proteins are better suited for acute infection rather than epidemiologic surveys (Christensen et al., 2002). The recommended assay by National Institute of Health is based on a 55 kDa recombinant protein of ORF2 that should ensure conformational epitopes are available (Tsarev et al., 1993). Peptide based assays have also been developed, where short immunogenic peptides are coated on the plate (Coursaget et al., 1994). ELISA assays based on recombinant proteins have shown to generally be more sensitive than assays based

on synthetic peptides (Mast et al., 1998). There are however no standards when designing an assay, and in the case of HEV there have been no guidelines from WHO. A wide variety of assays are available commercially as well as many assays developed in-house. Many of these build on similar principles, but may use different strains of HEV or have practical differences (Bendall et al., 2010). This can have an effect on the prevalences reported by different laboratories as sensitivity and specificity of the different assays do not compare. In Table 1 a small excerpt of reported seroprevalences are shown. Within Europe values as low as 0.3% in Greece (Stefanidis et al., 2004) and up to 53% in France (Mansuy et al., 2011) have been reported using different assays. In a recent study three commercial assays were used to analyze sera from 200 healthy healthcare workers and found 4.5%, 18.0%, and 29.5% of the samples positive for anti-HEV IgG using three different assays (Wenzel et al., 2013). A similar study was performed by Bendall et al. (2010), also concluding that assay variation was great, but also noted that the profile of subjects sampled also had a large impact on seroprevalence as well as the year of sampling.

8 Results

8.1 Manuscript 1 - Detection of a hepatitis E virus variant in farmed mink in Denmark

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Accepted for publishing in Emerging Infectious Diseases Journal

8.1.1 Abstract

Hepatitis E virus is a zoonotic virus with pigs as the primary animal reservoir. Recently, variants have been found in rats, bats and trout indicating that HEV variants are plentiful and widespread in many species. Here we report the finding of a novel HEV variant in farmed mink (*Neovison vison*).

8.1.2 Introduction

Hepatitis E virus (HEV) is one of the main causes of acute liver inflammation in humans. It belongs to the family *Hepeviridae* and is a non-enveloped RNA virus with a positive sense genome of approximately 7.2 kb. In 1997, HEV was discovered in pigs (1) and several studies have since shown that HEV is endemic in pigs and that pigs probably serve as an important animal reservoir and has traditionally been divided into 4 primary genotypes (G1-G4). G1 and G2 have exclusively been found in humans. G3 has been found globally in a wide range of mammals including humans, pigs, deer, rabbits and mongoose. G4, like G3, has an animal reservoir and have been found in humans, pigs and wild boars (2).

Along with the human and porcine variants, avian HEV (aHEV) has also been characterized and is wide spread globally and has been proposed to consist of three genotypes (3). During the last three years, a number of novel HEV variants have been described in red fox cutthroat trout, rat, bat and ferret (4-8). All new variants were clearly different from HEV G1-G4, aHEV and from each other. HEV is highly prevalent among pigs in Denmark with 92% of the herds being serological positive and approximately 50% of the investigated herds had pigs positive for HEV RNA (9). As HEV is highly prevalent in Danish swine, animals fed with offal from Danish slaughterhouses will be exposed to HEV. Production of mink fur is a significant industry in Denmark and mink are routinely fed a mixed diet often including swine offal. Inappropriate heat treated swine offal has previously been shown to be the source of swine related influenza A virus infection in mink (10, 11). Thus, the aim

of the present study was to investigate if Danish mink are infected with HEV G1-G4 or other HEV variants by screening feces and tissue samples collected from domestic and wild living mink.

8.1.3 The study

Initially, 85 fecal samples from farmed mink were screened by nPCR, detecting a broad panel of HEV variants (6). One sample was found positive and subsequent sequencing and phylogenetic analysis revealed that this virus represented a new HEV variant. To screen more samples for the presence of this new virus, a specific real time RT-PCR assay was developed (see appendix). The initially tested 85 fecal samples and an additional 233 fecal samples from farmed mink, together with liver and fecal samples from 89 wild-living mink were tested with this new and more sensitive assay. A total of four positive samples, all from farmed mink, were identified. In addition, screening with a HEV real time RT-PCR assay (9) specific for G1-G4 found none positive. The mink found to be infected by HEV were all submitted for diagnostic examination with a history of diarrhea in the herd. Three of the four submissions were from herds suffering from mink enteritis virus (MeV). Furthermore, the mink were diagnosed with hepatic lipidosis, aleutian mink disease virus (AMDV) and catarrhal enteritis (table, appendix).

The four samples positive for the novel HEV variant were collected between 2008 and 2011 on herds across Jutland, Denmark with a minimum distance of 80 km between the herds. The four PCR products obtained by the nPCR assay, covering a region of 261 bp of the RdRp-gene, were cloned and sequenced (Accession no. KC802090, KC802091, KC802092 and KC802093). The sequences were 98-100% identical with only one non-synonymous mutation resulting in a neutral amino acid change from isoleucine to valine (figure, appendix). The high homology in this region is not surprisingly since the gene encodes the RNA polymerase. Initial attempts were made to uncover a larger fragment by primer walking, but limited amount of material prohibited this.

The phylogenetic relationship of this novel mink HEV variant with variants found in other animals was analyzed based on the 261 bp fragment (Fig. 1). The mink HEV variant clustered together with HEV variants found in ferrets and rats which grouped in a separate branch clearly distinct from other previously described HEV variants. At nucleotide level, the mink HEV variant was approximately 65% identical with the closest classical HEV genotype (G3 and G4) and 76% and 69% identical with ferret and rat HEV viruses, respectively. At the amino acid level the homologies were more pronounced showing approximately 87% and 78% identity with ferret and rat HEV variants, respectively. The observed grouping of the HEV reference sequences included in the analysis was identical with previously performed phylogenetic analysis on full length sequences (12).

8.1.4 Conclusion

A variant of HEV was detected in four Danish farmed mink on four geographically distinct locations in a three year period which indicate that the virus has been circulating among Danish mink for a longer period. Phylogenetic analysis showed that the virus was clearly distinct from, but closely related to, ferret and rat HEV variants, recently reported from Germany and US (6, 7, 13).

It has not been possible to infect primates with rat or avian HEV variants (13, 14). Thus, due to the phylogenetic resemblance of mink HEV with these non-zoonotic HEV variants, there are no indications of mink HEV being able to infect humans, although no human samples have been tested specifically for this virus. The zoonotic potential of HEV has only been documented in the case of genotype 3 and 4 which were not found in mink, but taking the relatively high HEV seroprevalence in humans into consideration, the possibility of other variants being zoonotic and cross reacting with HEV genotype 1-4 in serological assays cannot be ruled out.

The mink in this study were from herds suffering from MeV hepatic lipidosis, AMDV and catarrhal enteritis, all factors that could explain the conditions of the mink infected with HEV (15). However, it cannot be ruled out that the mink HEV variant contributed to the clinical signs of the mink HEV

positive animals. To determine if the virus is indeed capable of inducing clinical signs in mink, experimental infection needs to be performed. However, the rat and ferret HEV variants induced almost no histological signs in rats post experimental infection and the ferrets were described as not showing overt clinical signs (7, 13). So far, only chicken infected with aHEV and humans infected with HEV G1-4 have been described to be clinically affected by HEV infections. There is a possibility that the HEV variants recently reported in a variety of different species, including the one reported here, will evolve into disease-causing pathogens in animals and possibly also humans.

Acknowledgements

We would like to thank Mariann Chriél for supplying the wild living mink samples and the Ministry of Food, Agriculture and Fisheries of Denmark (DFFE) for funding (project number 3304-FVFP-09-F-011).

Author Bio

Jesper S. Krog is a Ph.D student at the National Veterinary Institute of Denmark. The work presented here is part of a thesis clarifying the zoonotic aspects of hepatitis E virus in Denmark.

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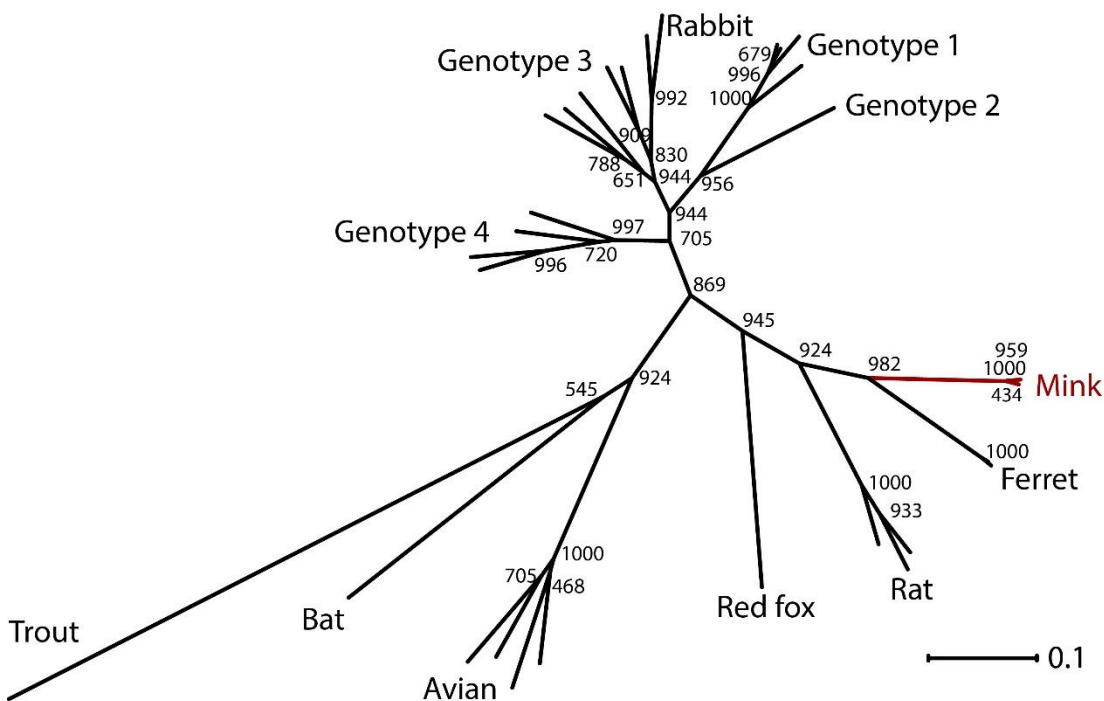


Fig. 1. The phylogenetic tree show the relationship between the novel mink HEV, other HEV variants and the four known HEV genotypes based on 261 bp of the RdRp gene. The CLC Main Workbench software was used for the phylogenetic analysis (CLC bio, Aarhus, Denmark). Alignments was made using MUSCLE algorithm and phylogenetic tree by using distance-based method with the Neighbour-Joining algorithm and bootstrap value 1000. Phylogenetic analysis with other methods showed similar results.

8.1.6 Technical appendix

8.1.6.1 nPCR

The nested PCR (nPCR) assay was performed as described by John et al., 2010 except that in this study the primer concentrations were 10 μ M in the first round and 100 μ M in the second round of the PCR.

8.1.6.2 Real time RT-PCR

A real time reverse transcriptase PCR (real time RT-PCR) assay that specifically detected this novel mink HEV variant was developed based on the sequence obtained from the first positive sample achieved by the nPCR assay. The nPCR product of this positive sample was cloned in pCR4 vector using the TOPO TA cloning kit (Invitrogen, Nærum, Denmark), hereafter referred to as pMINK. The real time RT-PCR assay was designed based on the Primer Probe Energy Transfer (PriProET) chemistry which allowed a few mismatches within the probe. The primers and probe were synthesized at Eurofins MWG Operon (Ebersberg, Germany). The unlabeled forward primer Mink-Fw 5'-CCAGAATGGTGCTTCTATGGTGAT-3' had a calculated T_m of 63.6 °C. The labeled reverse primer Mink-Rev 5'-FAM-AATTGTTCTGCGAGCTATCAA~~ACTC~~-3' had a calculated T_m of 62.5 °C. The labeled probe Mink-Probe 5'-GCCACACCTGCCGGGTCTTTGAAAACGATTT-ATTO633-3' had a calculated T_m of 75.0 °C. The location of primers and probe can be seen in TA figure 1. The real time RT-PCR was performed using QIAGEN OneStep RT-PCR kit (QIAGEN, Hilden, Germany) in a total volume of 25 μ l including 4 μ l extracted RNA, 100 nM Mink-Fw, 500 nM Mink-Rev and 500 nM Mink-Probe. The assay was run on a Rotor-Gene Q real time PCR cycler (QIAGEN) with the 72 tube rotor and channel settings at 470 nm for excitation and 660 nm for acquisition. The temperature profile was divided into three segments with a reverse transcription (RT) step, a PCR cycle and a melt analysis (MA). RT: 48 °C for 30 min, 95 °C for 15 min. PCR: 40 cycles of 94 °C for 15 sec, 55 °C for 1 sec with signal acquisition and then an additional 14 sec at 55 °C, 72 °C for 20 sec. MA: 95 °C for 45 sec followed by ramping the temperature from 45 to 80 °C in 1 °C increments and a 7 sec halt and signal acquisition at each step. The gain-optimization was set to automatic before first acquisition on tube 2. Data analysis was performed on the Rotor-Gene Q Series Software ver. 2.0.2 (QIAGEN) and the quantitation threshold was set at 0.01. The PCR performance was tested by a standard curve performed on a tenfold serial dilution of the constructed pMINK plasmid showing a PCR efficiency of 76 % ($R^2=0.999$), an eight \log_{10} detection span and an approximately detection limit at cycle threshold (Ct) 39. The melt analysis showed a T_m of 72 ± 2 °C across the dynamic

Results

range. The sensitivity was tested on a tenfold serial dilution of a positive mink fecal sample and compared with the nPCR assay. The real time RT-PCR assay detected the 10^{-3} dilution whereas the nPCR assay only detected the 10^{-1} dilution. All mink samples were run in duplicates with the duplicate being a 1:10 dilution of the original RNA extraction to exclude the influence of PCR inhibition.

8.1.6.3 Results

An alignment of the four sequenced mink HEV strains and the position of the developed mink HEV real time PCR assay can be seen in TA figure 1. A summary of the routine diagnostics analysis and the real time RT-PCR results for the four positive samples are summarized in TA table 1. One mutation was found in the probe region (TA figure 1) of the real time RT-PCR assay in sequence 1119 and 574, which was consistent with the lower temperatures of the melting analysis (TA table 1)

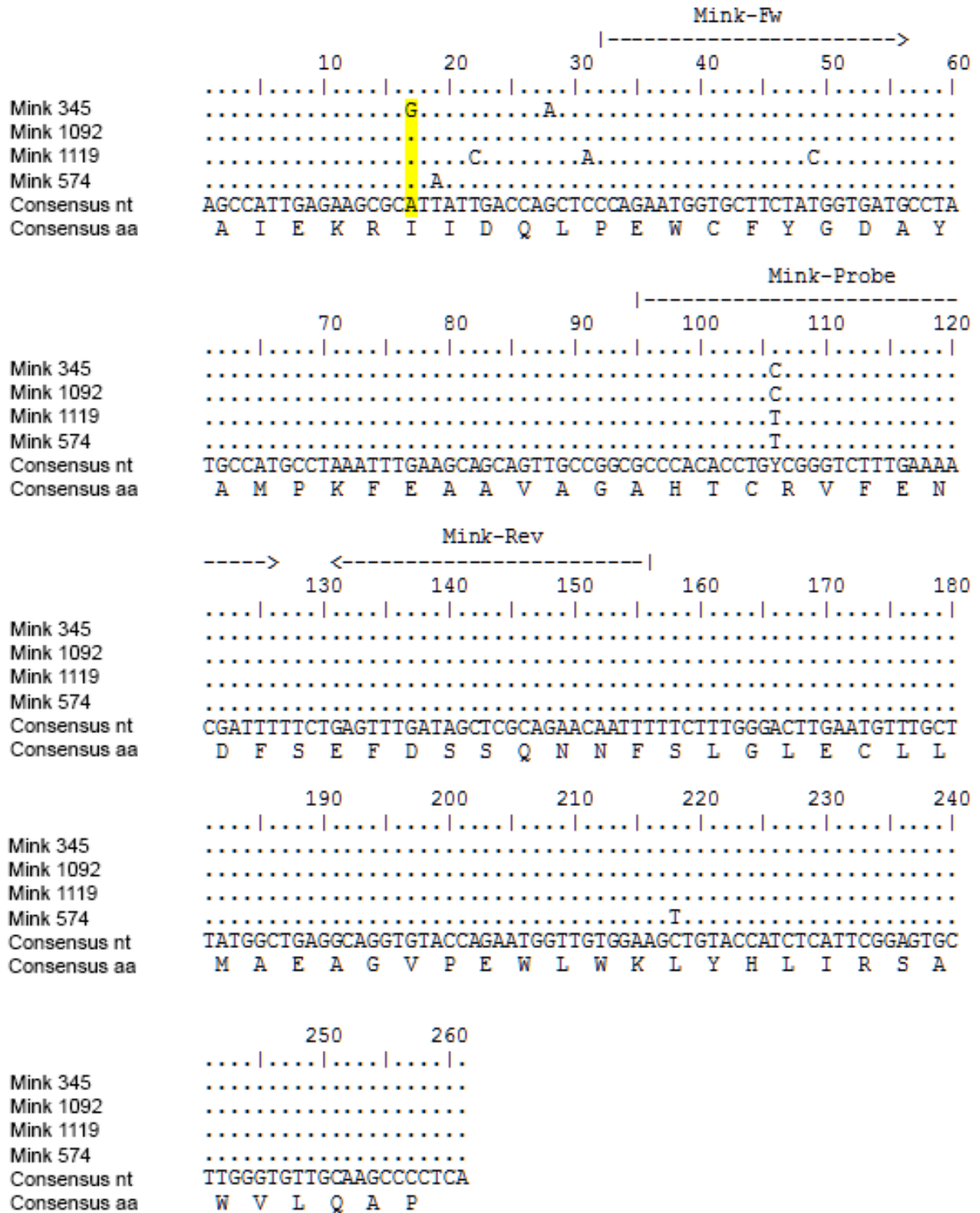
Results

TA table 3: Characterization of the four HEV positive samples.

Sample	Year	Sex	Age	Pathology	Histopathology	Diagnosis	C _i	MA
345	2008	F	-	Liver: yellow, enlarged.	Liver: Massive diffuse lipid vacuolization. Small intestines: Diffuse catarrhal enteritis.	- hepatic lipidosis - catarrhal enteritis - MeV not tested	25.1	73.2
1092-4*	2010	F	5 mth	Liver: yellow.	Liver: Lipid vacuolization, stasis. Small intestines: Catarrhal enteritis.	- hepatic lipidosis - catarrhal enteritis - MeV diagnosed in herd		
1092-5*	2010	M	5 mth	Liver: enlarged, hemorrhage, yellow. Spleen: enlarged, hemorrhage.	Liver: Massive lipid vacuolization, stasis, hemorrhage. Small intestine: Catarrhal enteritis.	- hepatic lipidosis - catarrhal enteritis - MeV diagnosed in herd	32.3	73.7
1119	2010	M	5 mth	Enteritis	Liver: Moderate to severe lipid vacuolization, stasis Small intestine: Dilatation of intestinal crypt cells, microabscesses squamous cells, shortening and fusion of intestinal villi.	- hepatic lipidosis - MeV diagnosed in subject and in herd	26.8	68.8
574	2011	M	3 mth	No macroscopically changes	Liver: No abnormalities Small intestine: Diffuse catarrhal enteritis.	- catarrhal enteritis - MeV diagnosed in herd	31.5	69.0

* The fecal sample from 1092-4 and 1092-5 were pooled hence only one C_i and MA value were recorded.

Results



TA figure 1: Alignment of the four positive mink HEV variant sequences obtained by the nPCR assay. The arrows indicate the primers and probe of the real time RT-PCR assay. The non-synonymous mutation is highlighted.

8.2 Manuscript II - Leaching of viruses naturally occurring in pig slurry to field drains and their correlation with other microorganisms

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Submitted for publication

8.2.1 Abstract

The amount of animal manure used in modern agriculture is increasing due to the increase in global, animal production. The contamination of the aquatic environment by root zone leaching of zoonotic pathogens from manure-treated fields has hitherto been overlooked. Pig slurry is known to contain zoonotic bacteria such as *E. coli*, *Salmonella* spp., *Campylobacter* spp. and viruses such as hepatitis E virus and group A rotavirus. Coliform bacteria, present in manure, have previously been shown to leach into tile drain. This poses a potential threat to aquatic environment and may also influence the quality of drinking water. In the present study, pig slurry was applied to a tile drained field and the leaching of six different microorganisms, *E. coli*, *Enterococcus* spp., somatic coliphages, hepatitis E virus, porcine circovirus type 2 and group A rotavirus, naturally occurring in the slurry, was investigated. All six microorganisms leached through the soil entering the tile drains situated in 1 meters depth. The leaching pattern of group A rotavirus differed substantially from somatic coliphages, otherwise used as indicator for microbial contamination. Furthermore, group A rotavirus was detected in groundwater up to two months after pig slurry application. The detection of viruses in drainage water and groundwater poses a potential human health risk as water reservoirs used for drinking water may be contaminated with zoonotic pathogens.

8.2.2 Introduction

While the threat of contamination by nutrients leaching from manure-treated fields is well recognized, the threat by leaching of zoonotic pathogens from the manure has received much less attention. Livestock manure is commonly used in modern agriculture as fertilizer. In the United States, livestock excrete approximately 500 million tons of manure annually (1) and an estimated 26 million ton of livestock manure was spread on Danish farmland in 2011 (2). The livestock manure contains nutrients and organic matter used to enhance soil properties and thus crop production, but may also contain a variety of zoonotic pathogens (3-5). Animal pathogens with potential negative impact on human health (zoonosis) include, among others, rotavirus group A (RV-A), hepatitis E virus (HEV), norovirus (NoV), *Salmonella* spp., *E. coli* O157:H7 and *Cryptosporidium parvum* (4). With the emergence of avian and swine influenza there have been an increased surveillance and focus on zoonotic viruses. The transmission of virus between mammals through the environmental reservoirs is, however, poorly understood. In the non-industrialized part of the world, hepatic viruses such as hepatitis A virus (HAV) and HEV causes many waterborne epidemics (6). In the western world, HEV was previously regarded as a travel related illness. HEV genotype 3 has since been discovered in pigs worldwide (7), and is now considered endemic in pigs in many European countries and North America and as the main reservoir for locally acquired HEV. The prevalence of anti-HEV antibodies in humans range between 2 - 52.5% (8-11). Detection of HEV in wastewater from urban areas has been reported in European and North American cities (12) indicating that HEV may be present in the water environment. Another virus with zoonotic potential is RV-A. RV-A mainly infects younger animals and children, and is the primary cause of children hospitalization due to gastroenteritis (13). RV-A has proved to be extremely stable in pig slurry storage tanks, with a reduction in infectivity of one log-unit taking over six months (14).

Enteric viruses and bacteria have been linked to disease outbreaks originating from contaminated water sources (15-20). Therefore detailed knowledge on the transport of microorganisms through soil is important in order to protect groundwater supplies from contamination. *E. coli* O157:H7 and *Campylobacter jejuni* present in manure resulted in a large waterborne disease outbreak by contamination of the drinking water system of Walkerton, Canada (21). The most plausible route was rapid horizontal transport in fractured bedrock. Similarly, drinking well water in a restaurant in Wisconsin was associated with illness caused by NoV (22). Zoonotic viruses such as HEV originating from pig slurry could pose a similar public health risk if transported to water bodies, including

drinking water reservoirs. Therefore, it is necessary to determine the travel distances and survival times of viruses in soils for risk assessment, management of contamination, and public health protection (23).

Transport of microorganisms through soil depends on e.g. soil type, precipitation and manure constituents as well as the size and surface properties of the microorganisms, with preferential transport through macropores having a major impact on the microbial leaching (24-29). The ability of the microorganisms to survive in the soil environment will depend on factors such as type of microorganisms, temperature, pH, moisture and composition of the indigenous microflora (30, 31).

Field studies have shown that transport of slurry constituents through soil to tile drains is possible and can occur shortly after slurry application (32-35). Field studies have mainly focused on fecal indicator organisms, e.g. *E. coli* and enterococci as well as bacteriophages used as model organisms for viruses (36-39), while studies on waste-associated human viruses, providing valuable information on the removal of these pathogens through the vadose zone (22, 40), are limited. Due to the risks associated with applying zoonotic viruses in environmental studies, bacteriophages have been used as a model for leaching of zoonotic viruses through soil (41-43).

Many countries assess the microbiological quality of water based on bacterial indicators such as enterococci and fecal coliform and total coliform bacteria, but such bacteria are often poor indicators of viruses (44, 45). Enteric viruses have been recognized as the causative agents in gastroenteritis outbreaks caused by water that have met bacteriological standards (46). Over 100 types of pathogenic viruses have been described to occur in water that has been contaminated with fecal material (47). Therefore, the use of nonpathogenic viral indicators of fecal contamination, e.g. coliphages, can be an important tool in public health studies, when tracing sources of groundwater contamination (48).

Thus, there is need for studies that are designed to measure the leaching of zoonotic viruses normally present in animal slurry. The main objective of the present study was to assess the risk of viruses from different families, such as HEV and RV-A, leaching into the aquatic environment when manure is applied to fields in accordance with current regulations. Porcine circovirus type 2 (PCV2) was included as this virus is ubiquitous in swineherds and highly persistent in the farm environment (49). In addition, the purpose was to compare the leaching capabilities of *E. coli*, *Enterococcus* spp., somatic coliphages, HEV, PCV2 and RV-A, and lastly, to evaluate if somatic coliphages is an appropriate model organisms for pig originated viruses under natural field conditions.

8.2.3 Materials and methods

Test field site. The experimental site was located at Silstrup south of Thisted in northwestern Jutland, Denmark (56° 56′ N, 8° 39′ E). The field is a part of the Danish Pesticide Leaching Assessment Program (50). The field was 17,100 m² (1.71 ha) and the terrain sloped gently 1-2°. The site was located on a glacial moraine of Late Weichselian age and has been exposed to weathering, erosion, leaching, and other geomorphologic processes for about 16,000 years (50). The soil was a sandy clay loam (14.6% clay, 11.6% silt, 67.7% sand and 4.1% organic matter) with pH 7.1 and a porosity of 0.42 cm³ cm⁻³ (50). The soil was heavily fractured and bioturbated and at 0.6 m below ground surface (bgs) 400 biopores/m² has been estimated. The drainage system in the field consisted of five parallel field drains running from south to the north. The five drains were connected to a transverse collector drain from which drainage water samples were collected. The tile drains were installed at an average depth of 1.1 m and an interspacing of approximately 17-18 m. Conventional agriculture had been practiced at the site during the previous 27 years and red fescue (*Festuca rubra* L.) was grown on the field during the study period. The field was encircled by a grass covered buffer zone being 18 m wide to the north and west, 10 m to the east and to the south 7 m of buffer zone was supplemented by a 3 meter paved road. Groundwater table, the minimum and maximum air temperature and soil temperature (30 cm bgs) was recorded on an hourly basis. Precipitation was measured at the site using a tipping bucket rain gauge system.

Sampling of pig slurry and drainage water. The pig (*Sus scrofa domesticus*) slurry was supplied by a local farmer. At the 5th of October 2011, the pig slurry was homogenized in the storage tank for approximately 1 h, using a slurry agitator (Kimidan Multimixer, Denmark). A total of 49 ton (29 ton ha⁻¹) of homogenized pig slurry was applied in bands on the soil surface by trailer hosing. The pig slurry was tested for the presence of somatic- and F-RNA coliphages, *E. coli* and *Enterococcus* spp., *Salmonella* spp. and the viruses, swine influenza virus (SIV), porcine parvovirus (PPV), HEV, RV-A, and PCV2 as described below. The microorganism *E. coli*, *Enterococcus* spp., somatic coliphages, HEV, PCV2 and RV-A was present in the pig slurry and therefore selected for analysis in the leaching study (Table 1). Drainage water was sampled flow proportionally (ISCO 6700 sampler, Teledyne Isco Inc., US) with sampling frequency being set by experience (51). Subsamples of 200 ml were thus collected for every 3000 l of drainage water flowing in the tile drains. Following the onset of

Results

heavy rainfall events, drainage water was sampled flow proportionally for approximately one day. To obtain weighted average concentrations for each storm event, the microbiological analysis was performed on pooled water samples containing all the subsamples collected during the heavy rain event. The “typical” events were defined as events causing the water level and accumulated flow rate within the preceding 12-hour period to exceed predefined levels that depended on the month of the year. A predefined water level rise and accumulated flow rate were set on base of experience (51). Additionally, field samples were collected from screens located at 1.5 - 2.5 m bgs from a monitoring well located in the buffer zone. Horizontal wells were installed at 3.5 m bgs, perpendicular to the tile drains of the field, and connected to a well in the western buffer zone. Samples from both well types were collected on a monthly basis. The day before a sampling, the wells were purged ensuring sampling of fresh water. The collection of water samples was conducted until 5th of January 2012. All samples were kept in a cooling box and transported to the laboratory where analysis for somatic coliphages and viable indicator bacteria were initiated within 12 h. Water samples for virus analysis were immediately frozen at -80 °C in 50 ml tubes.

Chemical analysis. Chemical analysis of slurry and drainage water samples was initiated within 24 h after sampling. Weekly collected drainage water samples were analyzed for the content of dissolved organic carbon (DOC) (52), total dissolved phosphorus (53) and total phosphorus (53) which include total dissolved- and particle associated phosphorus. In addition, pH was measured in water samples and slurry using a pH meter (PHM220; Radiometer, Denmark). Slurry samples were analyzed for dry matter, total-N, NH₄-N, phosphorus and magnesium at the OK Laboratory for Agriculture, Viborg, Denmark (<http://www.oklab.dk/>).

For the analysis of DOC, water samples were immediately filtered through a Whatman glass fiber prefilter (Whatman GmbH., Germany) and a 0.45 µm cellulose membrane filter (Whatman GmbH., Germany) and 15-20 ml of sample was transferred to a vial and adjusted to pH 2.5 using an Metrohm 848 Titrino Plus titrator (Metrohm AG, Switzerland). Measurement of DOC in triplicates was done using a Shimadzu TOC-VCPH analyzer (Shimadzu Scientific Instruments, Columbia, US). Drainage water to be analyzed for total dissolved phosphorus (53) was filtered through a Whatman glass fiber prefilter (Whatman GmbH., Germany) and a 0.45 µm cellulose membrane filter (Whatman GmbH., Germany) followed by acidification with addition of 1 ml of 4M H₂SO₄ per 100 ml water sample. The absorbance was measured at 880 nm using a Perkin Elmer Lambda 20 UV/Vis Spectrophotometer (Perkin Elmer, USA). Water sample for detection of particle associated phosphorus (53) was processed likewise but without the filtration step.

Fecal bacterial indicators. In both slurry and water samples, the fecal indicator organisms *E. coli* and *Enterococcus* spp. was enumerated by direct plating in triplicate on selective agar plates with a detection limit of 1 CFU ml⁻¹. Water and slurry samples were 10-fold diluted in Maximum Recovery Diluent (Oxoid, Hampshire, United Kingdom). Concentration of *E. coli* was determined on Brilliance *E. coli*/coliform Selective Agar (Oxoid), where colonies appear as typical indigo blue colonies after incubation at 37°C for 21±3 h. The concentration of *Enterococcus* spp. was determined as the number of typical red-maroon colonies on Slanetz and Bartley medium (Oxoid) following incubation at 44°C for 48±4 h (54).

Somatic coliphages. Somatic coliphages is a group of bacteriophages with the ability of infecting *E. coli* via the cell wall and belongs to four different families (55). Somatic coliphages were analyzed in triplicates with a detection limit of 1 PFU ml⁻¹ by plaque assay according to ISO 10705-2 (56). Briefly, slurry and water samples were 10-fold serially diluted in Maximum Recovery Diluent (Oxoid) and enumerated by the double-agar layer method. The host strain *E. coli* ATCC 13706 was grown in Nutrient broth (Oxoid) at 37°C for four hours. From the 10-fold diluted samples, one ml were mixed with one ml broth culture of the host strain and three ml soft agar consisting of 70% Blood agar base (Oxoid) and 30% Nutrient broth (Oxoid). The mixture was gently mixed and spread on a well-dried Blood agar base plate (Oxoid). Plates were incubated at 37°C for 18 h and clear zones (plaques) (PFU) were counted. Slurry was filtered through 0.45 µm pore size filters (Sartorius, Goettingen, Germany) before mixed with the soft agar when high bacterial background flora was expected.

Viruses. Prior to concentration of viruses, the pH of slurry and water samples was adjusted to pH 7 using NaOH and then clarified from debris by centrifugation at 4,000 rpm for 30 min at 4°C. To precipitate virus, 40 ml of the supernatant was transferred to tubes containing 0.7 g NaCl (Sigma-Aldrich, Brøndby, Denmark) and 3.2 g polyethylene glycol (Fischer Scientific, Slangerup, Denmark) and vortexed. The samples were placed on a shaking bed over night at 4°C followed by centrifugation at 10,000 rpm for 90 min at 4°C. The supernatant was discarded and viral nucleic acid was purified from the pellet using NucliSENSE reagents and the miniMag platform (bioMérieux, Herlev, Denmark) according to the manufacturer's protocol. The nucleic acid was eluted in 100 µl Raze free water.

HEV was detected by real time RT-PCR assay as described by Breum et al. (2010) using a final primer and probe concentration of 500 nM for HEV2-R and HEV2-P and to 100 nM for HEV2-F. The standard curve was prepared from plasmids containing the target gene of the assay. The

Results

amplification efficiency of the assay was 88% with a slope of -3.64. The detection of PCV2 was accomplished with the assay described by Hjulsager et al. (2009). The standard curve used to read viral load was made by spiking negative fecal samples with plasmid. The amplification efficiency of the assay was 82% and the slope -3.86. For detection of RV-A, the primer and probes used in the assay along with the PCR cycling settings was adopted from Pang et al. (2007). The assay was modified by the use of RNA UltraSense (Invitrogen, Nærum, Denmark) and RT-qPCR analysis was run on the Rotorgene Q real time PCR cyler (QIAGEN, Hilden, Germany). The standard curve was made from a serial dilution of RNA extracted from RV-A cultivated in MA104 cell line. The amplification efficiency of the assay was 92% and the slope -3.54. The standard curves were not used for absolute quantitation but to compare concentrations for each microorganism separately. Based on the dilution series made for the standard curve, a detection limit above Ct 38 for all three assays was applicable. As not all assays were absolute quantitative the concentration was calculated as RT-PCR units (RT-PCRu) with one unit defined as the lowest possible detectable dilution. The concentration of the individual water sample was calculated based on the individual standard curve. These values are therefore not directly comparable between the analyzed microorganisms.

Data analysis and statistical methods. The removal rate (λ) which defines the amount of microorganisms removed by passing through 1 m of soil was calculated using equation 1. The leaching of all microorganisms was normalized with the initial concentration detected in pig slurry (C_0). The depth (d) was set to the location of tile drain, i.e. 1.1 m bgs, and the removal rate was calculated based on the highest concentration (C_{max}) recorded in the event samples as proposed by Pang et al. (2009).

$$\lambda = - \frac{\log_{10}\left(\frac{C_{max}}{C_0}\right)}{d} \quad (1)$$

Recovery of microorganisms from pig slurry was calculated in three different ways, based on the maximum concentration detected in drainage water samples (C_{max}), in all event samples i.e. where the amount of microorganisms found in each event sample was summed, and all weekly samples, i.e. total amount of microorganisms detected in all weekly samples collected during the study period.

The statistical analysis was performed on log-transformed normalized data by permutation test with main effect on leaching differences of microorganisms and on days of sampling. The simulated P-values for the corresponding permutation tests on F-test values were calculated using R statistical

software suite version 3.0.0 with the ImPerm package version 1.1.2. Significance level was set at $P = 0.05$.

Pearson product-moment correlation coefficients were derived to assess the association between microbiological and environmental variables such as DOC, total dissolved- and particle associated phosphorus. Pearson coefficient was calculated using Excel version 15.

8.2.4 Results

Climate conditions. In the study period, running from 5th of October 2011 to 5th of January 2012 the total precipitation amounted to 286 mm. During October drainage runoff only occurred during four precipitation events. The following month of November proved to be relatively dry with only 49.5 mm precipitation compared to the 108 mm average for November recorded in the period 1961-1990 on site. This resulted in an entire month devoid of drainage runoff. At the end of November and start of December heavier rain resumed drainage runoff. The experiment ended in January with large amount of precipitation and drainage runoff (Fig. 1E). The air temperature in the study period varied between -2.6°C and 15.4°C and was relatively high for the season with the three primary months having only a total of three subzero days. The average number of subzero days in the study period was 20 (Fig. 1F). Soil temperature at 30 cm bgs was below 15°C for the entirety of the study.

Leaching of microorganisms. Initial analysis of water collected from drains and groundwater monitoring wells both fourteen days and one day prior to pig slurry application, showed no presence of *E. coli*, *Enterococcus* spp., somatic coliphages and of the viruses HEV, PCV2 and RV-A. The leaching of microorganisms is illustrated in the breakthrough curve (BTC) during the study period with the concentration detected in drainage water normalized against the initial concentration found in the pig slurry (Fig. 1). The initial breakthrough and relative concentrations of microorganisms is shown for weekly and event samples collected from the drains (Fig. 1B and 1C).

E. coli and *Enterococcus* spp. was detected in the first of three event samples caused by intensive rainfall during the first week (Fig. 1C). This rain event happened on the day after application of pig slurry and the concentration of *E. coli* and *Enterococcus* spp. in drainage water was 30 CFU ml^{-1} and 27 CFU ml^{-1} , respectively. *Enterococcus* spp. was detected again in the event sample collected 59 (2011-12-03) and 92 days (2012-01-05) after application of pig slurry. These event samples were

Results

both collected during very heavy rainfall (16.8 mm and 15 mm, respectively) with a concentration of 0.3 CFU ml^{-1} , essentially at the detection limit of the assay. *E. coli* and *Enterococcus* spp. was also found in the first weekly sample with a mean concentration of 13.5 CFU ml^{-1} and 5.7 CFU ml^{-1} , respectively (Fig. 1B). In the second and third weekly sample collected during the first and second week of December, *E. coli* was detected with 0.6 and 0.3 CFU ml^{-1} , respectively, which is nearly a 5-log-unit reduction compared to the concentration in the pig slurry added to the soil surface two months earlier. *Enterococcus* spp. was only detected in the first weekly water sample while all subsequent weekly samples tested negative for *Enterococcus* spp. (Fig. 1B). The removal rates of *Enterococcus* spp. and *E. coli* were comparable at 3.1 log m^{-1} and 3.3 log m^{-1} , respectively.

The abundant somatic coliphages was detected in the first five consecutive event samples from October 6th to November 26th. The removal rate of only 2.2 log m^{-1} was calculated based on the first event sample that had the highest concentration of viable coliphages at $1.4 \times 10^3 \text{ PFU ml}^{-1}$. This was the lowest removal rate of any microorganism assayed. The somatic coliphages reached the detection limit of the assay at the fifth event sample at the end of November. Somatic coliphages had a concentration of 345 PFU ml^{-1} in the first weekly sample, but was subsequently not detected in weekly samples.

HEV was detected in only the first event sample and correspondingly in the first weekly sample with both measurements close to the detection limit at 5 and $9 \text{ RT-PCRu ml}^{-1}$, respectively. The removal rate calculated based on the event sample was 3.1 log m^{-1} . Like HEV, the detection of PCV2 in water samples was low, ranging between $17 - 30 \text{ RT-PCRu ml}^{-1}$. PCV2 was detected in the first and second event sample along with the first weekly sample. The removal rate was 3.4 log m^{-1} which was similar to that of HEV.

RV-A was by far the most abundant of the viruses detected in the pig slurry with $3.81 \times 10^5 \text{ RT-PCRu ml}^{-1}$ (Table 1). RV-A was detected in the four first consecutive event samples and the concentration of RV-A increased in the drainage water over these four events (Fig. 1C). A removal rate of 2.7 log m^{-1} was calculated based on the fourth event where the highest concentration of RV-A was detected. RV-A was also detected in weekly samples collected between 2011-10-12 and 2011-12-16, but absent in the following sample collected on 2011-12-22, then reappeared in the next sample collected during very heavy rainfall in the start of January, yielding high flow in the tile drains and a significant rise in groundwater levels. The same phenomenon was observed for enterococci. The RV-A concentration of the later samples were essentially at detection limit with $2 \text{ RT-PCRu ml}^{-1}$.

Well samples and groundwater. During the study period, the groundwater table fluctuated at the level of the tile drain, but the groundwater level was always higher than the level at the time of slurry application (Fig. 1E). None of the microorganisms were detected in the water samples obtained from the vertical monitoring and the horizontal wells before application of pig slurry (Fig. 1D). Low concentrations of *E. coli* (0.4 CFU ml⁻¹) was detected in the vertical monitoring well at the start of December (day 57) while *Enterococcus* spp. was found at the start of November (day 29) and start of January (day 91) with similar low levels of 0.4 and 0.7 CFU ml⁻¹, respectively, corresponding to a 5-log-unit reduction compared to the measured concentration in pig slurry (Table 1). Somatic coliphages were detected in the water sample collected in November and December from the vertical monitoring well, both at low amounts, 1.2 and 0.7 PFU ml⁻¹, corresponding to more than a 5-log-unit reduction as compared to the pig slurry. RV-A was detected in both the vertical monitoring well and the deeper horizontal well at the first sampling on November 3th. The following month, a small decrease in the concentration of RV-A in the vertical monitoring well and an above tenfold reduction in the horizontal well was observed. In January, RV-A was no longer detected in the horizontal well and barely detected in the vertical monitoring well balancing on the detection limit of the assay (1.3 RT-PCR u ml⁻¹). Neither HEV nor PCV2 were found in any of the wells.

Microorganisms and slurry constituents. All six microorganisms were detected in water samples from the tile drains in the first event sample, and correspondingly also in the first weekly sample, (Fig. 1) while different leaching profiles between the microorganisms ($P = 0.04$) were observed during the study period. The leaching profiles of HEV, PCV2, somatic coliphages and *Enterococcus* spp. were very similar ($P = 0.31$) and showed a steep decline in concentration after the first week. Similar leaching profiles were observed between *E. coli* and RV-A ($P = 0.07$) and grouping them against the other four microorganisms showed that they were significantly different ($P = 0.01$).

During the study period, pH of the individual water samples, DOC, total dissolved phosphorus and particle associated phosphorus content was also monitored in the weekly drainage water samples (Fig. 1A). A strong correlation of all analyzed microorganisms except RV-A to particle associated phosphorus and DOC was found (Table 2). Conversely, RV-A was the only microorganism correlating strongly to the dissolved phosphorus (Table 2). No correlation between the six microorganisms and the pH of the drainage water was observed.

The recovery of the microorganisms in the tile drains depended on the time of sampling and was associated with rain events (Table 3). The recovery which was calculated based on the event sample

with maximum concentration of microorganism in tile drains, was generally at 0.03% - 0.04% for all microorganisms except the RV-A and somatic coliphages that had a recovery of 0.13% and 0.34%, respectively, which was also reflected by their lower removal rate. The recovery of the microorganisms based on the all the event samples were comparable to the recovered concentration based on the maximum concentration sample except for RV-A (Table 3). Generally the recoveries calculated on the two abundant microorganisms RV-A and somatic coliphages had far higher recoveries than the less abundant, indicating a difference in leaching according to the original concentration in the pig slurry. The recoveries based on the individual microorganisms in all the event samples, were generally similar when compared to the weekly samples.

8.2.5 Discussion

Leaching of microorganisms. We observed breakthrough of all six microorganisms in drainage water one day after pig slurry was applied on the field. Transport studies conducted at the Silstrup field have previously shown a high degree of preferential flow of chemical compounds with a travel time of 69 days to the drainage system, if the transport was mainly through the soil matrix (32). The leaching patterns of the microorganisms were interesting in that they appeared to have different leaching profiles. Somatic coliphages, HEV, PCV2 and *Enterococcus* spp. appeared immediately in drainage water in maximum concentrations followed by a rapid decrease, while leaching of *E. coli* and RV-A was observed for an extended period. The leaching of microorganism through soil depends on the extent of their retention in the soil as well as their ability to survive in the soil environment (61, 62). In structured soils, most studies of microbial transport generally show a rapid movement of bacteria and viruses due to preferential flow through macropores, e.g. naturally occurring cracks, fractures, earthworm holes, and channels formed by plant roots resulting in an early breakthrough, thereby bypassing much of the soil matrix (24, 63). Similar leaching pattern have been reported for phages and *E. coli* in field studies (31, 37). Differences in leaching between *E. coli* and enterococci have been ascribed to a stronger interaction between *E. coli* and soil particles (35). In the present study, the removal rate was similar for *E. coli* and enterococci. However, *E. coli* was detected for a prolonged period in the weekly samples which could indicate slower migration due to a higher interaction with soil as also observed for RV-A. When comparing the abundant RV-A and somatic coliphages a clearly distinct leaching profile was seen in the weekly samples, and interestingly, an

opposite leaching profile was observed in the first four event samples. RV-A concentration increased in drainage water during the first two weeks, whereas the concentration of somatic coliphages decreased. These very distinct leaching profiles of the two microorganisms, indicates that RV-A interacts with the soil particles within the soil matrix, whereas the somatic coliphages were rapidly transported through the soil via preferential flow. This indicates that somatic coliphages were inadequate indicators for RV-A and probably other similar viruses.

The high concentration of RV-A detected in the shallow screen of the vertical monitoring well and the detection of RV-A in the deeper horizontal well was unique for RV-A. None of the other microorganisms were detected in the horizontal well (3.5 m bgs). The difference in the detected levels of RV-A and somatic coliphages in the vertical monitoring well could be caused by the faster transport of somatic coliphages, i.e. the coliphages had passed the vertical monitoring well at time of sampling, whereas RV-A peaked at time of sampling. Another explanation is that a higher amount of RV-A leaches and for a longer period resulting in the elevated levels observed in the groundwater screen. The detection of RV-A in this deeper well is of major concern, since RV-A is a known cause of enteric disease in humans and animals and zoonotic transmission have been described at several occasions (13, 64). In addition, *E. coli* and *Enterococcus* spp. were also detected in the monitoring well. Shallow wells have previously been reported to be particularly susceptible to contamination of fecal bacteria from agricultural activities in clay rich sediments (65). The occurrence of fecal coliforms in tile drainage water in field experiments has been reported previously (31, 66, 67). In our study a recovery of 0.03 – 0.06% was found which is in accordance with results of similar field studies (31, 35). This is further corroborated by the *E. coli* recovery obtained in a leaching experiment performed on intact soil cores using soil of similar composition (42). The recovery of HEV and PCV2 were comparable to that of *E. coli* and *Enterococcus* spp., whereas the recovery of RV-A was similar to that of somatic coliphages.

Several factors have been ascribed to enhance migration of microorganisms through soil e.g. straining, pH, precipitation and manure constituents (24, 68). The viruses HEV, PCV2, and RV-A have been reported to have a diameter of approximately 32, 17 and 70 nm, respectively, whereas the somatic coliphages ranges from 24 – 200 nm (69). The slower leaching of RV-A could be due to size. However previous reports have indicated that straining have a larger effect on bacteria (0.5-2 µm) than on viruses, as viruses generally are smaller than the pore size of the soil, hence straining on virus due to size should be negligible (70).

Results

Isoelectric point of the total particle surface is another important factor that may impact leaching of microorganisms (71-73). The isoelectric point of two different strains of group A rotaviruses have been reported with different results. An isoelectric point of 8.0 was measured on the simian rotavirus SA-11 (74) and more recently, a porcine rotavirus with an isoelectric point of 4.5 was reported (75). The exceptional high isoelectric point of RV-A reported by Butler et al. (1985) could explain the difference in leaching pattern of RV-A compared to other microorganisms observed in this study, as the net positive charge of RV-A at neutral pH would allow for adhesion on the negatively charged soil particles. The low isoelectric point of 4.5 is in the range of many enteric viruses and phages (76) which at neutral pH gives a negative net charge of the particle and would result in repulsion from the negatively charged soil particles. Even though the overall charge of the particle is negative, there can still be local areas of positive charge, thus a larger particle will allow for more positively charged areas, capable of interactions with soil particles (72). In the work by Dowd et al. (1998) it was also shown that phages less than 60 nm in diameter were dependent on isoelectric point for adhesion, whereas size became the determining factor for adhesion properties of particles larger than 60 nm in diameter. This correspond very well with our data on viruses, where we observed a retention of the larger RV-A particle (70 nm) compared to the much smaller HEV (32 nm) and PCV2 (17 nm) particles.

Precipitation has proven to be an important aspect in facilitating the transport of microorganisms through soil especially immediately after manure application as also observed in this study. In addition, we found that heavy rainfall after three months promoted the leaching of *Enterococcus* spp. and RV-A to tile drain and monitoring well. This is corroborated by a study showing that pathogen transport through soil to drinking water wells is promoted by heavy rainfall (77).

Manure constituents such as DOC have been shown to enhance leaching of microorganisms by competing with favorable adhesion sites on soil particles (25, 78, 79). Royer, et al. (2007) showed that when a field was used for foraging, surface application of manure resulted in immediate leaching of DOC indicating preferential flow, which was far less pronounced when manure was applied to a field with corn. In our study, the field was planted with red fescue resembling the foraging state and a rise in DOC levels after application of pig slurry was also observed. Leaching of all microorganisms except RV-A correlated well to the leaching of DOC and particle associated phosphorus i.e. phosphorous that is bound to particles and also phosphorous bound in microorganisms. Phosphorus is generally considered to be well adsorbed by the soil matrix, but it has been shown that particle associated phosphorus adsorbed to a lesser extent than dissolved phosphorus, thus indicating

preferential flow (81). The leaching of RV-A on the other hand had a high correlation with the dissolved phosphorus which could indicate a higher adsorption to the soil matrix.

In summary, all microorganisms showed preferential flow characteristic and leached to tile drains immediately after slurry application. However, RV-A and *E. coli* seemed to have a higher interaction with soil particles which resulted in prolonged leaching.

Environmental and water safety hazards. The survival of a microorganism is an important factor when assessing the possible risk of groundwater contamination when applying manure on fields. We were able to detect *E. coli* and somatic coliphages for at least two months, while *Enterococcus* spp. was detected through the entire study period. Cools, et al. (2001) reported the survival of enterococci to be higher than that of *E. coli* in multiple soil compositions at temperatures below 15°C and at different moisture contents, which is in contrast to Pourcher, et al. (2007) that reported no difference in survival of these two fecal indicators. However, in both studies enterococci and *E. coli* survived for more than two months. The survival of somatic coliphages has been reported to be high with detection of infectious somatic coliphages in soil for 143 days post manure application (84). The survival of somatic coliphages has been shown to correlate to viral genome quantification, but not to fecal indicator organisms which survived for a shorter period (85). However, we found that leaching of *Enterococcus* spp. exceeded that of *E. coli* and somatic coliphages which again exceeded that of HEV and PCV2. The presence of HEV in the water environment of the western world has recently been recognized (86). There have previously been reports of HEV in urban sewage samples from Europe and USA (12) and that it can survive for at least one month in sewage (87). Furthermore, HEV viruses have been shown to survive for nine weeks at fluctuating temperature in soil and for ten weeks at 37°C (88). Surveys have found HEV in shellfish, and a study in the United Kingdom showed a prevalence of 50 – 96% HEV RNA in shellfish from coastal areas (89). An epidemiological study of autochthonously acquired cases of HEV was linked to consuming water from a private drinking water well in France (90). The majority of privately owned wells resides in rural areas, and therefore in close proximity of farmland, where wells are in risk of microbial contamination from land application of fecal originated wastes (77, 91). Since, some viruses have been shown to be transported in water reservoirs for longer than 400 m horizontally and more than 60 m vertically (92), the leaching of pathogens into the groundwater, after spread of manure, could expose humans to these pathogens by migrating into wells. Combined with the high stability of some of these viruses, there is a real risk for these viruses causing clinical illness. The survival of PCV2 have proved to be high with PCV2 being one of the most stable porcine viruses even withstanding disinfectants (93) and

therefore difficult to eradicate in swineherds. The possibility that PCV2 circulates in the water environment could also contribute to the risk of introducing or reintroducing PCV2 into susceptible swine herds. RV-A have previously been detected in drinking water (94). RV-A potentially poses a large problem, primarily due to the very large amount of virus excreted from animals and humans, high stability in water and zoonotic potential (94). The survival of human rotavirus in river water and tap water has been shown to be at least 60 days at 4 – 20°C (95). Similarly, the time to reduce 90% of infectious calf rotavirus in distilled water and sewage was shown to be 73 and 84 days, respectively (96). In this field study we found that RV-A was detectable in groundwater and persisted there for at least three month following application of pig slurry and the soil temperature measured during the study period would also favor an extended persistence of the microorganisms.

Methodology. Comparing different microorganisms can be challenging due to the difference in available detection methods to assay each microorganism. Real time PCR was applied for detecting the genome of the porcine viruses, whereas phages and bacteria were detected by plaque assay and colony assay, respectively. These assays are not directly comparable as the plaque and colony assay accounts for viable phages and bacteria, versus the real time PCR assays that target the genomes of infectious and non-infectious virus particles. The study of enteric viruses by the use of qPCR have previously been performed in water samples, where infectivity correlated very well with qPCR detection (97) and a correlation between the survival of somatic coliphages and viral genome quantification has also been reported (85). In our study, no comparison was done on the exact number of viruses detected by different assays. Instead the concentration of each microorganism found in the drainage water was normalized with the initial amount detected in the slurry, as these were measured by the same assay. The normalized concentrations of the microorganism were then compared in relation to leaching pattern, recovery and log-reduction in depth.

Similar, detection of bacteria is faced by the challenge due to the differentiation of dead and live bacteria, and the differentiation of these from culturable and viable but non-culturable (VBNC) bacteria depending on the method employed. The use of culturing and plaque assay has its limitation in that it is no guarantee that all pathogens of the same family are equally suitable for culturing (98). Bacteria entering the VBNC- stage are unable to grow on traditional culture media (99), but the persistence of bacteria in soil does not seem to be promoted by entering the VBNC-stage (100). Discrepancy in the amount of viable and culturable bacteria compared to quantification of cell numbers based on DNA, which include culturable and non-culturable or dead cells, have been reported in environmental samples (101-103). In the current study, only viable *E. coli*, enterococci

and somatic coliphages naturally occurring in the slurry and adapted to this environment were employed.

Implication. This field study investigated the simultaneous transport of RV-A, HEV, PCV2 and somatic coliphages under natural condition along with the leaching of *E. coli* and *Enterococcus spp.* through soil. The result showed immediate leaching of all microorganisms to tile drain. *E. coli* and RV-A had similar leaching pattern but different from somatic coliphages, PCV2, HEV and *Enterococcus spp.* The data generated in the present study suggest that somatic coliphages can be used to model transport of some viruses in soil, e.g. HEV and PSV2 but not a suitable candidate of porcine RV-A. Only RV-A was detected in the horizontal well corresponding to leaching into the shallow groundwater making RV-A a possible risk due to its high mobility, stable nature, high copy number and high prevalence in pig and cattle herds suggesting that this virus should be monitored more closely considering its zoonotic potential. As drainage water is led into surface water reservoirs this could pose a health risk to humans and animals exposed to these water resources.

8.2.6 Acknowledgements

We would like to thank Jens Molbo, Lasse Gudmundsson (ongoing field monitoring and data preparation), Gitte Petersen, Nina Flindt (laboratory assistance) and Bo Markussen (statistical support).

The study was supported by the PATHOS project (From manure to freshwater – technology avoiding contamination with pathogens, hormones and pharmaceutical) (www.pathos.geus.net) supported by the Danish Council for Strategic Research (ENV 2104-07-0015), the EU-funded PathOrganic under the CoreOrganic ERA-net (project no. 1888) (www.pathorganic.coreportal.org), The Danish Pesticide Leaching Assessment Programme (http://pesticidvarsling.dk/om_os_uk/uk-forside.html) and the Ministry of Food, Agriculture and Fisheries of Denmark (DFFE) for funding (project number 3304-FVFP-09-F-011).

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Results

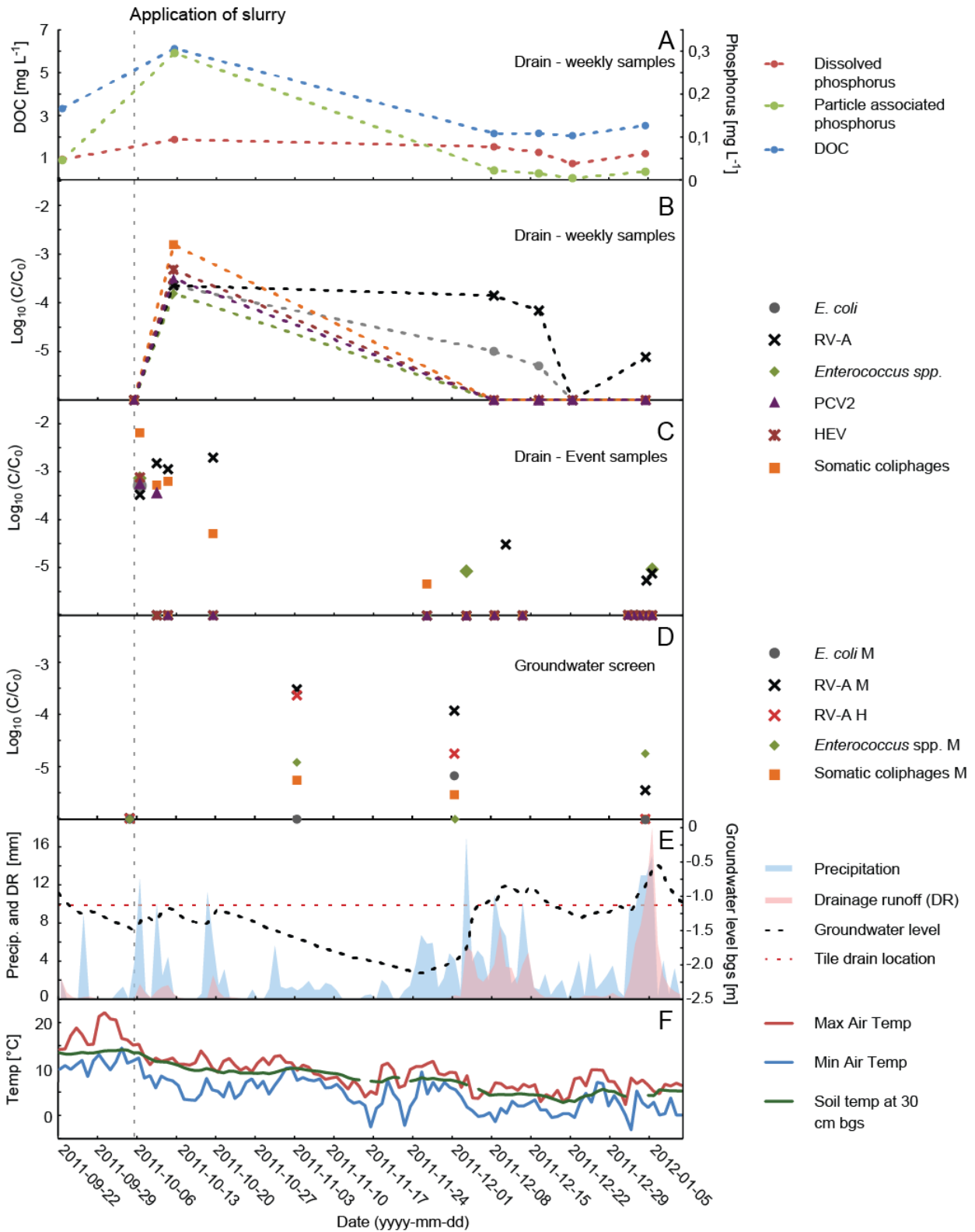


Fig. 1. Meteorological conditions and breakthrough curves for microorganisms and chemical constituents in drainage water in tile drains. Panel A shows leaching of phosphorus and DOC. Panel B shows the breakthrough curves of the assayed microorganisms in the weekly drainage

Results

water samples. Panel C shows the presence of microorganisms in tile drain at rain events. Panel D presents water samples collected monthly from both the vertical monitoring and horizontal well and only microorganisms above detection limit are indicated. Panel E shows the precipitation and drainage runoff (DR) together with the groundwater table. Panel F shows temperature variation in air and soil.

Table 1 Physical, chemical and microbiological properties of pig slurry

Dry matter	pH	Total-N	NH ₄ ⁺ -N	P	Mg	HEV	PCV2	RV-A	Somatic coliphages	<i>E. coli</i>	<i>Enterococcus</i> spp.
%		Kg/ton	Kg/ton	Kg/ton	Kg/ton	RT-PCRu/ml			PFU/ml	CFU/ml	
3.97	6.8	4.21	2.51	1.04	0.51	1.2×10 ⁴	5.4×10 ⁴	3.8×10 ⁵	2.2×10 ⁵	6.0×10 ⁴	3.6×10 ⁴

Table 2 Correlation between the six microorganisms and selected chemical constituents in weekly drainage water samples.

	pH	DOC	Particle associated phosphorus	Total dissolved phosphorus
HEV	0.44	0.96	0.99	0.74
PCV2	0.55	0.96	0.99	0.74
RV-A	0.46	0.69	0.79	0.94
<i>E. coli</i>	0.40	0.95	0.99	0.77
<i>Enterococcus</i> spp.	0.31	0.96	0.99	0.74
Somatic coliphages	0.39	0.96	0.99	0.74

Table 3 Recovery in percent of the six pathogens in percent in drainage water.

	HEV	PCV2	RV-A	Somatic coliphages	<i>E. coli</i>	<i>Enterococcus</i> spp.
Maximum concentration	0.04	0.03	0.13	0.34	0.03	0.04
All event samples	0.04	0.05	0.28	0.40	0.03	0.04
All weekly samples	0.11	0.07	0.20	0.35	0.06	0.04

8.3 Manuscript III - Enteric porcine viruses in farmed shellfish in Denmark

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Submitted for publication

8.3.1 Abstract

Bivalve shellfish are at constant risk of being exposed to pathogens as a consequence of contamination of the shellfish beds with human or animal waste originating from sewage treatment plants or slurry fertilized fields. Consumption of contaminated oysters and mussels are frequently reported as causes of disease outbreaks caused by norovirus or hepatitis A virus. Other zoonotic pathogens such as hepatitis E virus (HEV), rotavirus (RV) and *Salmonella* from livestock may also be transmitted to shellfish via this route. In this study, 29 pooled samples from commercial Danish blue mussels were tested for porcine pathogens and indicator bacteria *Escherichia coli* (*E. coli*). All samples tested negative for HEV, RV and *Salmonella*, whereas *E. coli* and the highly stable porcine circovirus type 2 (PCV2) were detected in eight and 13 samples, respectively. This is the first study to report the detection of PCV2 in commercial mussels. Based on the detection of PCV2 in clean areas with low prevalence of the normally applied fecal indicator *E. coli*, testing for PCV2 may be a more sensitive and robust specific porcine waste indicator in shellfish harvesting areas.

8.3.2 Introduction

Bivalve shellfish such as oysters, clams and mussels are recognized as important sources of foodborne pathogens. Bivalves take up nutrition from the surrounding water by filtrating up to 4.8 L/h (Carver and Mallet, 1990; Winter, 1973) and will simultaneously concentrate microbes if present (Burkhardt and Calci, 2000). Human pathogens can enter the shellfish beds in case of waste water managing system failures or in connection to flooding. Animal pathogens can contaminate the beds via runoff from fields applied with animal waste. A high number of food related outbreaks caused by contaminated bivalves has indeed been related to consumption of raw or lightly cooked oysters or mussels contaminated with noroviruses (NoV) (Westrell et al., 2010) or hepatitis A virus (HAV) (Pinto et al., 2009). Both NoV and HAV are shed in high amounts from infected humans, they are stable in the environment and the infectious dose is very low (Koopmans and Duizer, 2004; Teunis et al., 2008). Other viruses with zoonotic potential, such as hepatitis E virus (HEV) and rotavirus (RV), are also prevalent in production animals and are shed in large amounts in feces. HEV causes acute self-limiting hepatitis in humans similar to HAV (Cacopardo et al., 1997; Koizumi et al., 2004; Renou et al., 2008; Said et al., 2009) and is highly prevalent among Danish pig herds where 92% of herds have animals with antibodies against the virus (Breum et al., 2010). Group A rotavirus (RV-A) is excreted in feces from a range of production animals including bovines and pigs and may also have zoonotic potential (Fischer et al., 2005; Martella et al., 2010; Midgley et al., 2012). Thus, shellfish produced close to land, where spillover with porcine waste can occur, may accumulate zoonotic enteric viruses and by that act as a vehicle for human exposures and subsequent diseases.

Currently, the application of slurry to farmland is tightly regulated in most countries, but failure to follow regulations or extreme weather conditions may nevertheless cause release of virus contaminated slurry into the surrounding water environment.

The hygienic control of fecal contamination in shellfish beds is based solely on the levels of the indicator bacteria *Escherichia coli* in shellfish meat according to the European directive 91/492/EC (Anonymous, 1991) and by fecal coliform in waters used for shellfish harvesting areas in the US according to the National Shellfish Sanitation Program issued by the FDA (FDA, 2009). These regulations have successfully reduced the number of clinical cases associated with bacterial infections caused by ingestion of seafood (Lees, 2000). However, the presence of bacterial indicators have been shown to be insufficiently correlated to the presence of enteric viruses (Lees, 2000). Additionally, these indicators do not provide information of the source (human or animal) of contamination.

Furthermore, the commercially applied “depuration”, a process where shellfish is placed in a tank of clean water to clear out pathogens, efficiently clear bacteria, but this process has limited impact on the clearance of viruses (Loisy et al., 2005; Love et al., 2010; Schwab et al., 1998). Consequently, half of the clinical cases caused by seafood consumption in i.e. New York, are now caused by viruses (Butt et al., 2004; Wallace et al., 1999).

Denmark has a substantial pig as well as shellfish production and is surrounded by water (Fig 1) and therefore there is a risk of shellfish being contaminated by viruses present in pig slurry. The primary aim of this study was to investigate the presence of the viral pathogens HEV and RV-A, in blue mussels produced near the coast of Denmark. Secondly, the aim was to evaluate the potential of the highly stable and pig specific porcine circovirus type 2 (PCV2), which is considered ubiquitous in swine herds (Kristensen et al., 2013), to serve as an indicator of porcine waste and determine the correlation between the porcine viruses and the presence of *E. coli* and *Salmonella* in mussels.

8.3.3 Material and methods

8.3.3.1 Samples

Twenty nine samples of blue mussels (*Mytilus edulis*), from 19 different Danish commercial harvesting areas (see Table 1) were collected by the Danish Veterinary and Food Administration (DVFA) during the official national control program of the fishermen’s own control program in 2008 and 2009.

8.3.3.2 Bacterial analysis

The mussels were tested for *E. coli* and *Salmonella* according the EU reference methods (Anonymous, 2002, 2005) within 48 hours post-harvest by the DVFA Regional Control Laboratory, North.

8.3.3.3 Viral analyses

To extract viral nucleic acid from mussels, digestive tissue (DT) from at least 10 animals originating from one or two neighbor harvesting areas was excised, pooled and comminuted by racer blades. Viral nucleic acid was extracted from 2.0 g sub-samples of DT according to the method included in the newly developed ISO TS 15216 standard Anonymous (2013), except that the entire amount of homogenized DT using three ml of lysis buffer and 140 µl magnetic beads was processed as described by Uhrbrand et al. (2010).

Results

To evaluate the extraction efficiency of viral nucleic acids from the mussel tissue, approximately 10^4 plaque forming units of mengovirus (MC_0), was added as internal process control to all portions of homogenized DT prior to proteinase K (PK) treatment. The relative recovery efficiencies and the inhibition during detection of HEV, RV-A and PCV2 in mussel DT, were determined in two independent runs using mussel DT from a confirmed negative sample and pig slurry previously shown to contain 1.2×10^4 , 5.4×10^4 and 3.8×10^5 PCR units ml^{-1} of HEV, PCV2 or RV-A particles, respectively. One PCR unit was defined as the highest dilution that tested positive by the assay. The recovery efficiencies were calculated as the differences in average Ct (ΔCt) values obtained from nucleic acid extracts of 140 μl pig slurry alone and 2 g DT spiked with 140 μl slurry prior to PK treatment. To determine the effect of PK treatment on the virus recoveries during nucleic acid extraction, Ct values obtained from virus detection in pig slurry by the inclusion and exclusion of PK prior to the nucleic acid extraction were compared.

The inhibitory effect of the mussel extract was calculated as the differences in Ct values obtained from testing 1 μl of slurry extracts alone, and spiked with undiluted and 10-fold diluted mussel extracts (4 μl).

Detection of viruses was carried out by Real time RT-PCR on a RotorGene Q (QIAGEN) using the RotorGene Q Series software 2.0.2. All samples were assayed in duplicates of undiluted and 10-fold diluted nucleic acid extracts. HEV was detected using the assay by Breum et al. (Breum et al., 2010) applying modified primer and probe concentrations, HEV2-R and HEV2-P (500 nM) and HEV-F (100 nM), and reaction conditions, denaturation (15 s), annealing (15 s) and elongation (20 s). From the 10 fold dilution series of a plasmid containing the target region, an amplification efficiency of 88% and a slope of -3.64 was calculated. RV-A and MC_0 were detected using the RNA Ultrasense One-Step qRT-PCR System (Invitrogen, cat number 11732-927) and the primers, probe and reaction conditions described by Pang et al. (Pang et al., 2004) for RV-A and by Pinto et al. (Pintó et al., 2009). The standard curve for RV-A was made from a serial dilution of RNA extracted from RV-A cultivated in MA104 cell line. The amplification efficiency of the assay was 92% and the slope -3.54. The detection of PCV2 was accomplished by the real time PCR assay described by Hjulsager et al. (2009). The standard curve was made by spiking negative fecal samples with plasmid. The amplification efficiency of the assay was 82% and the slope -3.86.

Pearson's product-moment correlation coefficients between bacteria positive values and viral positive values were calculated to determine the correlation between bacterial and viral contamination.

8.3.4 3 Results

8.3.4.1 Detection of pathogens and fecal indicators

All 29 mussel samples tested negative for HEV, RV-A or *Salmonella*, 13 samples (40.6 %) tested positive for PCV2 and eight samples (28 %) had detectable levels of *E. coli*. The process control, MC₀, was consistently recovered in all mussel extracts, indicating robust extraction performance. The recovery efficiency in the nucleic acid extracts of mussels varied according to the type of virus (Table 2) with a net reduction in viral genome detection corresponding to a 2.1, 0.5 and 0 log reduction of RV-A, HEV and PCV2, respectively. Based on the Ct values obtained from virus detection in slurry extracts compared to extracts of mussel homogenates spiked with slurry (Table 2), the efficiency in purification of HEV and PCV2 were consistently similar in the two extracts with maximum Δ Ct of 1.61 in the undiluted extracts. In contrast, the recovery efficiency of RV-A decreased considerably when purified from pig slurry alone, compared to mussel extracts spiked with pig slurry with a Δ Ct of 7.51 in undiluted extracts. To identify the cause of poor RV-A assay performance, loss of viral particles during PK treatment and inhibition during detection were studied. Unlike for PCV2, the PK treatment of pig slurry during nucleic acids extraction had a reducing effect of approximately 1 log on the detection of HEV and RV-A. However, as negligible Δ Ct-values between extracts of slurry and PK treated slurry was observed when testing 10-fold diluted extracts, the reduction in slurry extracts must be attributed to inhibition arising from PK treatment rather than loss of viral particles. To further test for inhibition, Ct-values obtained from virus detection in pig slurry extracts alone and pig slurry extracts spiked in mussel extracts were compared (Table 2). This corresponded to 1.1, 1.6 and 0.4 log inhibition of the RV-A, HEV and PCV2 assays, respectively, in undiluted extracts which could be reduced to 0.3, 0.8 and 0.3 log by testing for inhibitors in 10-fold diluted mussel extracts.

8.3.4.2 Correlation between pathogens and fecal indicators

The correlation between mussel samples positive for both PCV2 and the bacteria, *E. coli* and/or *Salmonella* were determined (Table 1). The bacterial content were in general very low (≤ 40 MPN *E. coli*/100 g flesh and liquid), except for sample 14 which contained very high amount of both *E. coli* (310 MPN /100 g) and PCV2 (Ct= 33.7). However, as only three of the 11 PCV2 positive samples were found positive for *E. coli*, there was no statistical significant correlation between the presence of PCV2 and bacteria in shellfish ($P = 0.43$). The geographic distribution of the PCV2 positive shellfish samples revealed that positive samples were localized to the fjord Limfjord in the northern part of Jutland and a bay area in the south-western part of Jutland, Denmark (Fig. 1).

8.3.5 Discussion

Here we demonstrate that a high fraction of mussels in Denmark contained high levels of PCV2. PCV2 is a pathogen that has only been detected in pigs and is not considered a threat to public health. The presence of a porcine virus in shellfish do, however, document that virus present in pig slurry has the potential to be accumulated in shellfish which is often consumed raw or lightly cooked. Interestingly, the zoonotic viruses RV-A and HEV were not detected in shellfish in the present study despite these viruses indeed are present in pig slurry in positive herds and the HEV prevalence in Denmark is more than 90% (Krog et al., submitted for publication 2013; Breum et al., 2010). Even though RV-A and HEV are stable in the environment, then PCV2 is one of the most resistant viruses known (Ansari et al., 1991; Balayan, 1997; Kim et al., 2009; Parashar et al., 2011; Welch et al., 2006). Nevertheless, RV has previously been detected in shellfish and experiments with virus-like particles have shown that RV have the potential to persist in shellfish (Abad et al., 1997; Hansman et al., 2008; Le Guyader et al., 2008). The persistence of HEV in soil has been compared to that of HAV. HEV survived for nine weeks at outdoor temperatures and for ten weeks at 37°C whereas HAV persisted for 8 and 13 weeks, respectively (Parashar et al., 2011).

The validation of the assays showed different performance against virus stability towards the rigorous treatment with PK, during viral nucleic acid extraction of mussels and inhibitors during detection, which could also explain why HEV and RV-A were not detected. The RV-A assay was pronouncedly affected by inhibition due to both the PK treatment and constituents left over from the mussel homogenate. Unlike RV-A, the PCV2 assay was not affected by inhibition and losses during nucleic acid extraction, whereas the HEV assay showed mild inhibition compared to RV-A. Especially the two log reduction of RV-A would indeed exclude weak positive samples from being detected. However, according to the *E. coli* monitoring of Danish shellfish harvesting areas a generally low fecal pollution rate have been demonstrated in connection to the national classification system with more than 95% of samples comply to class A-status each year,.

The lack of HEV detection in Danish mussel samples is in accordance with a surveillance of HEV in mussels (213 samples) from Thailand (Namsai et al., 2011), but in contrast to data obtained in United States and United Kingdom where two of 46 clam samples and 41 of 48 mussel samples, respectively, were found positive for HEV (Crossan et al., 2012; Hansman et al., 2008). In the study from United Kingdom, 26 samples were collected near an outlet of wastewater from a pig processing plant possibly supplying a constant outlet of HEV. The possible continuous delivery of this wastewater, could explain the high prevalence even if HEV does not persists for long in shellfish.

Results

The fact that PCV2, but not HEV, was detected in the Danish mussels could be due to a potential selective accumulation of PCV2 (Burkhardt and Calci, 2000). In a study by Sobsey et al. (1987) the depuration of HAV and poliovirus was significantly different with poliovirus being removed from the shellfish at a much higher rate than HAV. Furthermore, different species of oysters have shown different ability to accumulate and depurate viruses. As an example, the oyster *Crassostrea ariakensis* was shown to more efficiently accumulate NoV, murine norovirus 1 and HAV than *Crassostrea virginica* (Nappier et al., 2008). Moreover, a difference in accumulation and persistence between different strains of NoV in a single species of shellfish have been shown (Le Guyader et al., 2012). This can be explained by a difference in ligand/receptor affinity, meaning that certain shellfish species have receptors that bind certain strains of viruses. This is relatable to RV-A that has 14 different G-types referring to its VP-7 viral particle component and 22 P-Types referring to its VP-4 protein of the viral particle, making it possible that certain G and P types of RV-A is preferentially accumulated. In one study, the G and P type detected in oysters were not genotypes typically associated with pigs (Le Guyader et al., 2008). In a study on Japanese clams (*Corbicula japonica*) G3 and G4 were found (Hansman et al., 2008), which are two of the four primary genotypes found in pigs (Midgley et al., 2012). These are however also prevalent in humans (Santos and Hoshino, 2005).

A number of factors can contribute to the contamination of PCV2. The positive samples were exclusively found in confined water bodies such as fjords and bay area. Both serve as an outlet of streams making the contact area of agricultural farmland vast. The pollution from mainly agriculture (water drained from fields and spillover) and wastewater to Danish streams and fjords, have been a concern for decades because pesticides and nitrate has been shown to enter these reservoirs (Kronvang et al., 2003). Therefore it was not surprising to find PCV2 positive samples in these areas. Another possible factor is season. Positive samples were mostly detected in spring/early summer and in early fall, which is consistent with periods of the delivery of slurry to farmland. In particular, if we consider the samples harvested in early fall, they tend to be more positive (lower Ct) than samples harvested later in the season.

To our knowledge this is the first report of PCV2 in shellfish. More information is needed regarding accumulation and persistence of this type of viruses in blue mussels and other species of shellfish. However, based on the detection of high number of PCV2 positive samples in otherwise negative shellfish, PCV2 should be considered as a new sensitive indicator of porcine waste contamination of shellfish. This information can be used to evaluate the hygienic quality of shellfish, but also to rectify

problems with spill over from porcine industry and agriculture. PCV2 is not a virus which is of public health concern, but other single stranded circular DNA viruses such as TT-like viruses have been found in pigs and in humans so the interspecies transmission and possible reservoirs of this type of viruses should be monitored more closely (Kekarainen and Segalés, 2012; Li et al., 2010).

8.3.6 Acknowledgement

We would like to acknowledge Albert Bosch at the University of Barcelona for supplying the mengovirus. Resadije Idrizi for technical assistance, Charlotte K. Hjulsager for assistance with PCV2 analysis and the Ministry of Food, Agriculture and Fisheries of Denmark (DFFE) for funding (project number 3304-FVFP-09-F-011).

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Results

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Results

Table 1: Summarized data for all samples

ID	RV-A	HEV	PCV2 (Ct) ^a	<i>E. coli</i> ^b	Salmonella	Harvest date	Area	Production site
1	-	-	33.75 (0.22)	<20	-	03-04-2008	a	Fjord
2	-	-	34.72 (0.04)	<20	-	03-04-2008	a	Fjord
3	-	-	37.30 (0.35)	20	-	07-05-2008	b	Fjord
4	-	-	38.51 (0.61)	<20	-	07-05-2008	c	Fjord
5	-	-	38.78 (0.26)	<20	-	07-05-2008	b	Fjord
6	-	-	39.80 (0.18)	<20	-	07-05-2008	d	Fjord
7	-	-	-	<20	-	07-05-2008	d	Fjord
8	-	-	38.08 (0.06)	<20	-	07-05-2008	b	Fjord
9	-	-	-	40	-	11-06-2008	e	Fjord
10	-	-	-	<20	-	11-06-2008	f	Fjord
11	-	-	39.94 (0.47)	<20	-	25-06-2008	g	Fjord
12	-	-	-	20	-	26-08-2008	h	Fjord
13	-	-	39.13 (0.12)	40	-	08-10-2008	g	Fjord
14	-	-	33.70 (0.29)	310	-	29-10-2008	i	Bay area
15	-	-	36.91 (0.42)	<20	-	29-04-2009	j	Fjord
16	-	-	-	<20	-	29-04-2009	j	Fjord
17	-	-	-	<20	-	17-08-2009	k	Bay area
18	-	-	-	40	-	17-08-2009	k	Bay area
19	-	-	-	40	-	17-08-2009	k	Bay area
20	-	-	-	<20	-	17-08-2009	l	Ocean
21	-	-	-	<20	-	17-08-2009	m	Ocean
22	-	-	-	<20	-	18-08-2009	n	Ocean
23	-	-	-	<20	-	19-08-2009	o	Ocean
24	-	-	-	<20	-	19-08-2009	p	Ocean
25	-	-	-	20	-	19-08-2009	q	Ocean
26	-	-	38.13 (0.81)	<20	-	11-11-2009	r	Fjord
27	-	-	-	<20	-	11-11-2009	d	Fjord
28	-	-	-	<20	-	11-11-2009	s	Fjord
29	-	-	-	N.T	-	04-01-2010	?	Fjord

^a Ct = number of cycles to reach the detection limit. Number in parentheses is the standard deviation of the replication

^b MPN pr. 100 g muscle

Results

Table 2. Method performance. Viral recovery during nucleic acid extraction and inhibition during detection of viral genomes.

	Virus recovery, Ct Average (\pm SD)						Effect of PK ^a		Inhibition ^b	
	Slurry		Mussels		Δ Ct (mussel-slurry)		1:1	1:10	1:1	1:10
	1:1	1:10	1:1	1:10	1:1	1:10				
PCV2	33,85 \pm 1,30	36,33 \pm 1,72	33,61 \pm 1,65	36,29 \pm 2,11	-0,25	-0,04	0,89	-1,70	1,69	1,32
HEV	32,32 \pm 0,51	35,63 \pm 1,71	33,92 \pm 0,63	36,87 \pm 1,40	1,61	1,24	3,38	-0,39	5,84	3,11
RV-A	26,22 \pm 1,06	27,97 \pm 2,12	33,73 \pm 1,61	37,65 \pm 2,12	7,51	9,68	3,52	0,06	3,91	1,14

^aThe effect of PK treatment on virus detection in slurry extracts, Δ Ct of pig slurry treated with PK and untreated.

^bInhibition in extracts during detection, Δ Ct of mussel RNA spiked with slurry RNA and slurry RNA alone. 1:10 indicates dilution of the mussel RNA prior to spiking.



Fig. 1: Geographic distribution of samples. Samples positive for PCV2 are indicated by a red dot, negative samples are indicated by blue dot.

8.4 Manuscript IV – Longitudinal study of hepatitis E virus in pigs: From sow to grocery store

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Preliminary manuscript

8.4.1 Abstract

Pigs are considered the main reservoir for the human pathogen Hepatitis E virus (HEV). HEV is prevalent in swine herds globally, thus consumers will be exposed to HEV during the food chain if the virus is present in pigs at slaughter. The aim of the present study was to study the HEV infection dynamics from births to slaughter in 104 pigs from 11 sows in a single production system. Serum was collected from sows 2 weeks prior to farrowing and feces and serum were collected from pigs every second week from week 1 through week 17. At slaughter at week 20, feces and selected organs from 10 pigs were sampled. In addition, 73 commercial pig livers sold in grocery stores were collected. All samples were tested for HEV virus RNA by real time PCR and the serum samples were tested for HEV specific antibodies using a commercial ELISA.

Maternal antibodies (MatAb's) were present in all pigs and all pigs, but one, seroconverted to HEV in week 13-17. In total, 2/3 of the pigs tested positive for HEV virus RNA at least once during the study (on weeks 13, 15 and/or 17) and significantly fewer pigs with high level of MatAb's became shedders. In contrast, the level of MatAb's had no impact on onset and duration of shedding. HEV was detected in feces and in organs, but not in muscles, of 3 out of 10 pigs at slaughter, indicating that test of feces are indicative of HEV positivity in organs. One liver from a grocery store tested strongly positive for HEV representing a prevalence of 1.4%.

In conclusion, a high proportion of pigs in a HEV positive herd were infected and shed virus during the finishers' stage and a fraction of the pigs also contained HEV RNA in feces and organs at slaughter. Level of MatAb's had a positive impact on the prevalence of shedding animals and therefore sow vaccination may be an option to decrease the prevalence of HEV positive animals at slaughter, however, more studies are required to investigate this.

8.4.2 Introduction

Hepatitis E virus (HEV) is a zoonotic virus with domesticated pigs being regarded as the main reservoir. HEV comprises four genotypes, genotype 1 and 2, exclusively found in humans and genotype 3 and 4 with zoonotic features. Genotype 3 is spread worldwide in pigs and sporadic human cases are seen, while genotype 4 is found in both pigs and humans in Asia, but have recently also been isolated in Europe (Hakze-van der Honing et al., 2011). A high prevalence of HEV antibodies (Ab's) in swine herds was found in both contemporary studies performed in several countries and a Spanish retrospective study with samples dating back to 1985 (Breum et al., 2010; Casas et al., 2009; Garkavenko et al., 2001). The high HEV seroprevalence in older samples indicated that HEV has been present in pigs for decades and raises a concern for the risk of HEV entering the food production. Recent studies have highlighted the fact that consumers are indeed exposed to HEV since 0.8% to 11% of porcine livers bought in supermarkets were found to contain HEV specific RNA by PCR (Feagins et al., 2007; Kulkarni and Arankalle, 2008). Furthermore, HEV isolated from the commercial livers was infectious for pigs in an experimental trial (Feagins et al., 2007). In another study, a pig liver sausage, figatellu, which is traditionally eaten raw, where found to be the cause of hepatitis in a significant number of people who consumed it (Colson et al., 2010). In addition, a relatively high number of pigs have been shown to be infected with HEV at slaughter (Di Bartolo et al., 2011; Leblanc et al., 2010). Previous longitudinal studies performed in pigs revealed that most of the pigs became infected at 8-15 weeks of age, but still some of the pigs were positive at slaughter (Casas et al., 2011; de Deus et al., 2008; Meng et al., 1997). Maternal antibodies (MatAb) against HEV have been shown to be successfully transferred from HEV-Ab positive sows to offspring, but in a previous study comparing few animals in a single herd, the level of MatAb had no impact on the infection dynamic of HEV in the offspring. Thus, the protective role of IgG Ab's is presently unclear (Kanai et al., 2010).

The proven zoonotic potential of HEV in pigs combined with the relative high prevalence of HEV positive pigs in Denmark may potentially have a negative impact on the food safety of Danish pork products if the virus is present in Danish pigs at slaughter. Thus, it is essential to increase the knowledge of HEV infection dynamics in typical pig production systems. The aim of the present study was to study the HEV infection dynamics from births to slaughter with special focus on the impact of maternal antibody levels and the infectious status of individual pigs at slaughter. The dissemination of HEV in different tissues of natural infected pigs that shedded virus three weeks prior

slaughter and commercial pig livers sold in grocery stores were examined to elucidate and quantitate the zoonotic risk.

8.4.3 Material and methods

8.4.3.1 Longitudinal study design

More than 100 crossbred pigs were sampled every second week from birth until slaughter. The pigs were kept at the breeding unit until approximately 30 kg where they were moved to the finisher site situated approximately 16 km from the breeding herd. Before the study, the presence of HEV in gilts at the nursery site was confirmed by testing feces from 10 sows by a HEV specific real time RT-PCR assay (data not shown).

Selection of sows: Two weeks prior to farrowing, serum samples were collected from 58 sows and tested for HEV Ab's and HEV specific RNA, respectively. Based on the measured, normalized levels of HEV Ab's sows were divided into three groups; low ($1 < OD < 2$), mid ($2 < OD < 3$) and high ($OD > 3$). The group of low level Ab's comprised of 23 sows with a mean normalized OD of 1.38 (SD=0.27). The, mid and high level Ab's groups each included 17 sows, with mean normalized OD values of 2.44 (SD=0.24) and 4.50 (SD=1.47) respectively. The local farmer randomly selected four sows from each group to be included in the study. Just after farrowing, all piglets from the 12 sows were ear tagged with a unique number. If more than half of the piglets within a litter died, the sow and her piglets were excluded from the study.

Sample collection: One week after farrowing, blood sampling of all piglets was performed. Thereafter, both rectal swabs and blood sampling was performed every second week until week 17 for all piglets. The pigs were restrained either manually or with a snout break and 9 ML of blood collected directly into a plan container (venoject) by puncture of the jugular vein. Rectal swabs were collected by swapping a cotton swab at the rectal surface approximately 2-3 cm from the anus and then placed in a sterile container with 2 ML PBS. Samples were labeled and kept cooled during transport to the laboratory. Blood samples were then stored at 4°C prior to further processing the same day. Serum was extracted from whole blood by centrifugation at 3000 RPM for 10 minutes at 5°C. The serum fractions were then transferred into nunc tubes and stored at -80 °C until RNA extraction. Tubes containing the cotton swab and 2 ml of PBS was shaken at 300 rpm for 1 hour before pouring the liquid into 2 ml eppendorf tubes and stored at -80°C until analysis. Individual pigs were excluded from the study if more than two sampling dates were missed.

Selection of pigs for tissue sampling: Ten pigs, randomly selected among pigs that were positive for HEV RNA in feces at week 17, were separately necropsied at a laboratory facility situated 100 km from the herd. At the age of 20 weeks, the pigs were transferred to the laboratory alive and on a vehicle with no other pigs present. At arrival the pigs were killed by intra-cardiac injection of pentobarbiturate (50 mg/kg) followed by exsanguination by cutting the arteria axillaris. Tissue samples (3x3 cm) were immediately collected at necropsy and included tonsils, lung, kidney, spinal cord, gall bladder (intact), hepatic lymph node, colon with content, small intestines with content, mesenteric lymph node heart and the entire liver. Furthermore, muscle/meat samples were taken from shoulder, neck, pork loin, tenderloin, ham and diaphragm. From the colon and small intestines, contents were collected. The tissue was then rinsed off in cold PBS. In addition, bile was extracted from the gallbladder with a syringe and a small piece of tissue was excised and rinsed in PBS to remove the remaining bile. All samples were placed in labeled nunc tubes and stored at -80°C until analysis.

8.4.3.2 Commercial liver samples

Between March and May 2012, 73 packages of commercial pig livers, representing five different abattoirs, were purchased pairwise each week in six different supermarkets and one butcher shop in the vicinity of Copenhagen. According to information available on the packages the pigs were raised, slaughtered and processed in Denmark. All liver samples were excised and homogenized in Buffer RLT (QIAGEN) immediately after purchase. If RNA extraction was not performed the same day, the samples were stored at -80°C until further processing.

8.4.3.3 RNA extraction and PCR analysis

Automated extraction of viral RNA from the rectal swab supernatant was performed on the QIAasymphony SP system (QIAGEN) using the DSP virus/pathogen mini kit version 1 (QIAGEN, Cat no. 937036). The protocol used was complex 200 V5 DSP with an elution volume of 110 µl. Total RNA was extracted from the livers bought at grocery stores using RNeasy mini kit (QIAGEN).

HEV RNA was detected by real time RT-PCR as described by Breum et al. (2010) except that the concentration of the primers was changed to 500 nM for HEV2-P and HEV2-R and 100 nM for HEV2-F and the time settings used for the PCR cycling were adjusted (15 sec for denaturation and annealing, 20 sec for elongation).

8.4.3.4 Serological analysis

All serum samples were tested for HEV IgG using a commercial kit (PrioCHECK® HEV Ab porcine kit; Prionics). All samples having an OD value that exceeds the OD of the cut-off control (available in the kit) multiplied by 1.2 were regarded as positive. All OD values were normalized by dividing the OD of sample with the OD of cut-off control multiplied by 1.2, which eliminated plate-to-plate variations. Normalized OD values above one were considered positive. According to the information provided by the vendor, the assay had a sensitivity of 91% and a specificity of 94%.

8.4.3.5 Statistical analysis

The statistical analysis was performed using SAS 9.1. For determining the overall difference between the three groups, mixed linear model was used. This method allowed for missing datapoints from individual pigs. To evaluate the difference on a weekly basis ANOVA analysis was performed. Finally, to compare groups for difference in number of shedders χ^2 -test was applied. For all analysis the significance level was set at $P = 0.05$

8.4.4 Results

Initially, a total of 12 sows and 135 piglets were included, but during the study, 31 of the piglets, including one entire litter, either died or were excluded due to missing sampling points. Thus, data from a total of 104 piglets from eleven sows were included in the analysis.

8.4.4.1 Serology

Based on the levels of HEV Ab's prior to farrowing, the 11 sows were allocated to one of three groups with low, mid or high levels of HEV Ab, designated group 1, 2 and 3, respectively. Normalized OD values for HEV Ab levels of the included sows, numbers of piglets in each litter and in each group are listed in Table 1. All pigs, but one, seroconverted during the study (Fig. 1). Pigs in groups 1 and 2 showed similar serological profiles with OD values below the cut off until seroconversion that occurred between week 11 and 13 followed by a steady increase in IgG levels that lasted until the end of the observation period at week 17 (Fig. 1). Group 3 showed a different profile with positive IgG levels from birth until week 7 and group 3 pigs, like group 1 and 2, seroconverted between week 11 and 13 followed by a steady increase in IgG levels until week 17 (Fig. 1)

There was a clear effect of grouping between group 3 and group 1 and 2 showing significant difference in levels of IgG ($P = <0.0001$ and $P = 0.0006$, respectively). When comparing all three groups at each sampling point a significant difference was found from week 1 to 11, but not at week 13 to 17 (Fig. 1).

8.4.4.2 Real Time PCR

Of the 104 pigs included in the analysis, 66 pigs (63.5 %) were positive for HEV RNA in feces in at least one sample during the study period (Table 2). There was a significant difference in the number of shedders from approximately 73% for groups 1 and 2, to 45 % for group 3 ($P = 0.032$) (Table 2). No significant difference in the onset of shedding was observed between the groups ($P = 0.876$). None of the pigs excreted virus before week 13 and only 9 pigs from all three groups started to shedding at week 13 (Fig. 2). The majority of the pigs excreted HEV virus for the first time at week 15 whereas 6 pigs had onset of shedding at week 17. Of the 104 pigs, 23 (22%) had HEV in feces at two samplings and two pigs (2%) were positive at three samplings (weeks 13, 15 and 17) (Fig. 2).

8.4.4.3 Analysis of tissue from pigs at slaughter age

To analyze if different organs and tissues contained HEV at slaughter, 10 of the 26 pigs shedding HEV at week 17, approximately three weeks prior to slaughter, was random selected for analysis. The 10 pigs included 3, 5 and 2 pigs from groups 1,2 and 3, respectively. The HEV IgG profiles for the 10 individual pigs from birth until slaughter are outlined in Fig. 3A. Three of the pigs (1-1, 2-1 and 3-1) did not seroconvert until week 20, but both pigs 2-1 and 3-1 started shedding virus before week 15 (Fig. 3B). At slaughter at week 20, three of the 10 pigs, one from each group, were still positive for HEV RNA in feces at a level similar to that observed at week 17 (Fig. 3B). There was no significant difference in the HEV shedding pattern before week 17 for the three pigs positive for HEV at week 20 compared to the other seven pigs negative for HEV at week 20 ($P = 0.633$). Interestingly, only the three pigs with fecal shedding at week 20 were positive for HEV RNA in organs (Table 3). Only internal organs were found positive for HEV RNA while none of the muscle samples tested positive. The liver associated organs (liver, bile, gall bladder and hepatic lymph node (HLN)) were strongly positive for HEV RNA (low Ct) whereas lower levels of HEV RNA were detected in extra-hepatic organs such as lungs and tonsils.

8.4.4.4 Livers bought at grocery stores

To get an estimate of the prevalence of HEV positive livers, additionally 73 livers bought at grocery stores were tested for HEV RNA. One of the 73 livers (1.4%) were found to be positive for HEV RNA ($CI_{95\%}$ of]0 - 4.1%]). Subsequent sequencing and phylogentic analysis showed that it belonged to HEV genotype 3 clustering with other European strains (data not presented).

8.4.5 Discussion

Offspring from 11 sows with different levels of HEV specific antibodies were included in the present study. To investigate the efficacy of passive transfer and the subsequent effect of maternal antibodies on the HEV infection dynamic in the offspring, the 104 piglets were allocated to one of three groups based on the relative level of anti HEV antibodies measured in the sows, three weeks prior to farrowing (low, medium and high). MatAb's were detected only in the group of piglets from sows with high level of Ab's prior to farrowing, revealing a clear correlation between the levels of HEV Ab's in the sows and the maternal HEV Ab's in the piglets. This finding is in accordance with previous studies, which also confirmed that a certain level of antibody is required for effective transfer (Casas et al., 2011; de Deus et al., 2008; Meng et al., 1997). The differences between the piglets in the group with high level of MatAb's compared to the other groups were statistically different until week 13. Previous studies have confirmed that MatAb's against HEV wean at around weeks 9-13 (Casas et al., 2011; de Deus et al., 2008).

HEV RNA was detected in feces of pigs in the present study at week 13 and onwards. Thus, no shedding was detected in the pigs when housed in the sow herd since the pigs were moved to the finisher site at 30kg (weeks 9-12). Based on the presence of HEV Ab's in the sows and the detection of RNA in gilts in the sow herd prior to the study (data not shown), HEV was indeed present in the sow herd, but it is not clear if the piglets were infected by HEV just prior to being moved or if the pigs were infected after arrival at the finisher site. The fact that there was no effect of the level of MatAb's on the onset or duration of viral shedding support the hypothesis that the pigs were exposed to HEV relatively late i.e. after the MatAb's had weaned. On the other hand, if exposure took place after the pigs had been moved to the finisher site, it is difficult to explain why significant fewer pigs in the group with initially high level of MatAb's were found to become shedders compared to the other groups. A previous field study failed to show any effect on the level of MatAb's on the risk of becoming a shedder, however, that study was performed on very few animals (2 litters) and the pigs were infected very early (week 3-4) indicating a heavily contaminated environment (Kanai et al., 2010). Another field study detected RNA in feces of pigs starting in week 12-15 approximately 3-5 weeks after the MatAb's had weaned which are more in line with the findings in the present study (de Deus et al., 2008).

Seroconversion measured by a commercial HEV ELISA was observed in the present study in all pigs, but one, starting week 13 which is in accordance with development of IgG in previous studies (Casas et al., 2011; de Deus et al., 2008; Meng et al., 1997). Thus, also pigs that were PCR negative at all

Results

samplings seroconverted indicating that they indeed were infected or at least exposed to HEV but at levels below the detection limit of the PCR assay. The seroconversions coincided with the onset of shedding for most of the pigs. This was unexpected since IgG Ab's previously have been shown to develop 2-3 weeks after onset of viraemia (de Deus et al., 2008; Kanai et al., 2010). Detection of HEV viremia in the present study were attempted at the same sampling days as for the feces samples, but were unsuccessful even though different methods for RNA were tested and the assay previously has performed very well in detecting HEV RNA in serum samples from the field (Breum et al., 2010) and in an ring trial (unpublished results). The virus load of HEV in serum has, however, previously been shown to be significantly lower than in feces and the viraemia also seem to be of shorter duration than the fecal shedding (Kanai et al., 2010). Furthermore, in an experimental trial in pigs with intravenous exposure of homogenates of livers with different levels of HEV it was shown that the duration and level of viraemia were strongly correlated to the level of HEV present in the inocula (Feagins et al., 2007). Thus, a likely explanation for the finding in the present study, i.e. seroconversion that coincided with positive fecal samples, late infection and lack of detection of virus in serum, could be that the pigs at arrival to the finisher herd were exposed to relative low levels of virus at a relative high age, resulting in a limited infection which in turn led to level of viraemia below the detection limit, limited and delayed hepatic replication and by that delayed shedding which in turn coincided with seroconversion. This explanation also take into account that the duration of shedding in the present study was shorter than in previous studies in that HEV was detected only at one sampling in 75% of the pigs. Another contributing factor to the early detection of Ab's could be that the anti-porcine IgG conjugate included in the ELISA cross-reacted with IgM Ab's which normally develop earlier than IgG (Casas et al., 2011; Meng et al., 1997).

HEV RNA was detected in internal organs (intestine, lymphatic tissue, bile and liver), but not in muscles, in three out of ten pigs tested which are in accordance with previous findings (Feagins et al., 2007; Kanai et al., 2010; Leblanc et al., 2010). Interestingly, only pigs that tested positive in fecal samples at slaughter were positive in organs. This indicated that testing of pigs feces prior to slaughter could be used as an indicator of HEV contamination of internal organs. However, albeit all feces positive pigs were found to harbor HEV in tissue in one previous study (Leblanc et al., 2010), then the predictive value of a negative test may be limited since HEV previous has been detected in organs from pigs that tested negative in feces (Casas et al., 2011; Leblanc et al., 2010).

The detection of HEV in organs of slaughtered pigs correlated well with the finding that a single pig liver bought in a Danish grocery store was highly positive for HEV. One positive liver out of 73

indicated a prevalence of 1.4%, which is similar to results obtained in studies from the Netherlands, France and Southern Germany. A much lower prevalence were found in India (0.8 %) and Japan (2 %) (Bouwknegt et al., 2007; Kulkarni and Arankalle, 2008; Rose et al., 2011; Wenzel et al., 2011; Yazaki et al., 2003) whereas in the USA a much higher fraction of livers was found positive (11 %) (Feagins et al., 2007).

In conclusion, a high proportion of pigs in a HEV positive herd were infected and shed virus during the finishers' stage and a fraction of the pigs also contained HEV RNA in feces and organs at slaughter. Level of MatAb's had a positive impact on the prevalence of shedding animals and therefore sow vaccination may be an option to decrease the prevalence of HEV positive animals at slaughter, however, more studies are required to investigate this.

8.4.6 Acknowledgements

Markku Johansen, Pig Research Centre is thanked for help with sampling and details on the pigs, Lars Springborg for access to his herd; Birgitta Svensmark and Svend Haugegaard, Pig Research Centre Kjellerup laboratorium for help with sampling and necropsy and Stud.Ing Camilla Bitsch Larsen for help with analysis of livers

The study was supported by the Ministry of Food, Agriculture and Fisheries of Denmark (DFFE) (project number 3304-FVFP-09-F-011).

8.4.7 References

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Table 1. Grouping of piglets according to level of maternal antibodies

Group	low level Ab (group 1)				Mid level Ab (group 2)			High level Ab (group 3)			
Sow ID	3399	3545	3485	3681	3266	3699	3548	3552	3532	3292	3145
OD (norm.)	1.3	1.4	1.4	1.4	2.5	2.8	2.9	5.8	6.1	6.9	11.6
# Piglets (in study/born)	8/8	15/19	6/10	9/10	10/11	8/10	15/18	8/10	9/10	8/10	8/10
Total # pigs group	38				33			33			

Table 2. Number of first time shedding pigs in each group. Each pig is only represented in the week of the first registered shedding.

	Low	Mid	High	Total
Week 13	2	6	1	9
Week 15	21	17	13	51
Week 17	4	1	1	6
Total shedders	27/38 (73.7%)	24/33 (72.7 %)	15/33 (45.5 %)	66/104 (63.5 %)

Table 3. Detection of HRV RNA in tissue samples. Only pigs with positive samples were included.

	Pig 68	Pig 114	Pig 137
Feces	26.11	23.3	25.4
Small intestines (content)	27.9	27.0	-
Colon	-	-	-
Small intestines	-	-	-
Intestinal lymph node	37.2	-	38.3
Gall bladder	31.1	31.3	29.2
Bile	24.9	23.4	27.7
Liver	30.8	21.5	27.5
Hepatic lymph node	26.9	30.7	36.6
Kidney	-	-	-
Lung	34.1	34.7	35.3
Tonsil	38.8	-	38.3
Spinal cord	-	-	-
Muscles*	-	-	-
Heart	-	-	-

*Muscles included six different samples of muscle collected from parts of the pig often used for foods made for human. All samples were analyzed separately.

Results

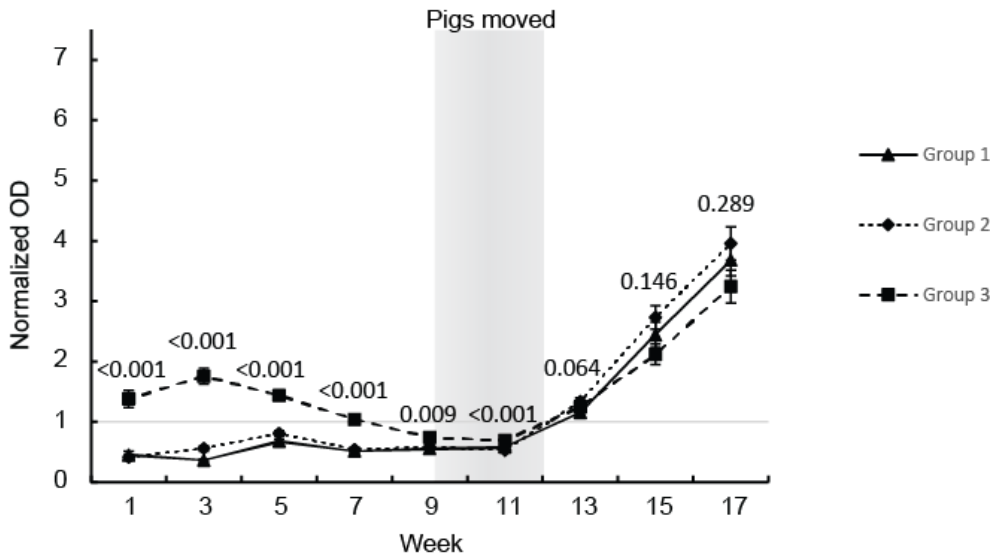


Fig. 1 Antibody (IgG) development. The values are expressed as mean value of normalized OD for the pigs in the three groups. The results of the statistical analysis of the difference between the groups (ANOVA) are indicated at each sampling point

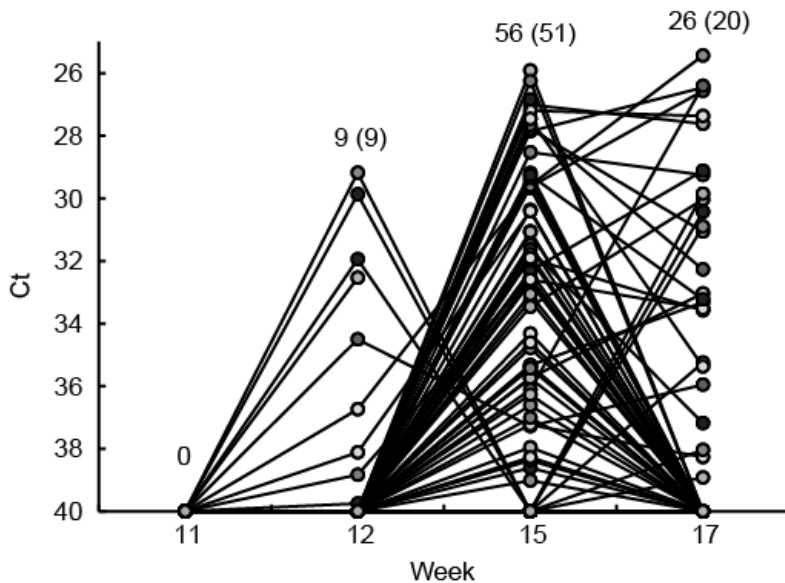


Fig 2. The fecal shedding of HEV from all pigs is shown with the Ct value detected by real time RT-PCR. Numbers indicate number of animals positive numbers (number of first time shedders).

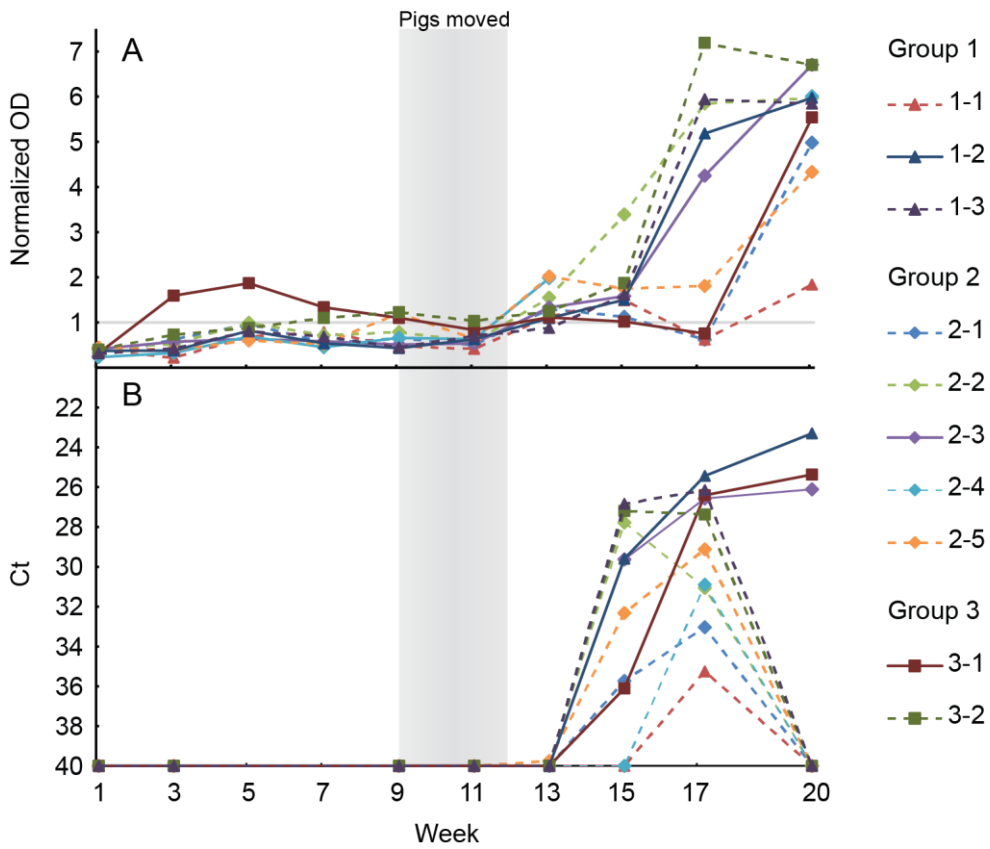


Fig 3. A: IgG measurements of the ten pigs necropsied in the sydy note that pig 2-4 have an incomplete set of data with serological samples for week 15 and 17 missing B: Fecal shedding of HEV in the ten pigs necropsied. Ct scale has been inverted and negative samples have been set at 40 Ct.

9 Discussion

The virus family of Hepeviridae is relatively new, and many new members have recently been discovered in multiple species. The zoonotic potential of these viruses is currently uncertain, but there is no epidemiological data that indicate zoonotic transmission of these new HEV like viruses. We identified a novel HEV variant in farmed mink without being able to connect the presence of the virus with pathologies. The lack of clinical signs is a common trait in many species infected with HEV variants and only humans and poultry are clinically affected. The widespread nature in different species of these HEV variants could indicate a high possibility of interspecies transmission, as recently shown between rats and Asian musk shrew (Guan et al., 2013). Thus, potentially these viruses may in the future cross the species barrier and become human pathogens as seen for HEV G3 and G4.

Christensen et al. (2008) showed a decreasing trend in HEV seroprevalences of the Danish population from 1983 (31.5%) to 2003 (20.8%). Public awareness to kitchen sanitation and the preparation of pork meat, garnered a lot of attention during the mid 1990ies due to serious cases of salmonella poisoning. The Danish government imposed further regulation in the herds and in the slaughtering practices to minimize the prevalence of salmonella in pigs and pork products, a mission that have been successful in reducing the occurrence of salmonella significantly (Ministry for food, 2013). Salmonella is, like HEV, an enteric pathogen therefore the increased focus on avoiding fecal cross contamination in the production of pork meat could have had a synergistic effect to bring down HEV contamination, thereby limiting the exposure of humans to HEV. Nevertheless, we and others have found a relative high prevalence of HEV in organs from pigs at slaughter despite the very limited fecal cross contamination that may occur in modern Danish slaughterhouses probably because the virus was present in the organs prior to slaughter. Thus, a way forward to decrease the prevalence of HEV positive pork products would be to decrease the prevalence of HEV positive pigs at slaughter. Indeed, we found a strong correlation between actively shedding HEV and detection of HEV in tissue samples at slaughter, but we also demonstrated that pigs receiving high level of maternal antibodies were less prone to shed virus. Thus, routine vaccination of sows may significantly decrease the number of HEV positive pigs entering the food production line. To completely prevent HEV infected pigs from entering the food chain, sterilizing herd vaccination would be necessary and this will not be possible with the present available vaccines. If the problem of late shedders (shedding at time of slaughter) are due to low infectious pressure in the herd that allow pigs to avoid being infected until late (15-17 weeks) in the

finisher period i.e. as we observed in our field study, an obvious solution would be to vaccinate the pigs with live HEV at an early age. However, this practice could pose a health problem for the persons handling the pigs. Furthermore, using live vaccine could have the opposite effect in that it could increase the number of shedders and thereby the viral load in pig slurry resulting in increased contamination of the water environment because of field application of pig slurry. We found a clear correlation between shedding of HEV in feces at slaughter and the presence of virus in internal organs. Eliminating these pigs from the food chain could have a great impact in reducing the direct exposure of humans to HEV originating from pigs. Thus, an effective measure to prevent HEV from entering the food chain would be to test fecal samples of individual animals prior to slaughter and then exclude organs from the positive animals from entering the downstream processing lines. This practice would, however, be costly, and hard to implement as high throughput detection methods of HEV in an active infection is largely based on PCR. Furthermore, our study on slaughtered swine included very few animals, so the strict correlation of feces and tissue has to be confirmed by others. Testing of feces samples may be used in the future in the case that some sensitive export markets demands HEV free meat. It is however difficult to compare these studies due to different conditions such as experimental infection vs. natural infections. As discussed above, control at the herd level by vaccination is a more plausible solution. A third approach is to bring more awareness to the problem of undercooking especially internal organs of pigs. Thus, the French government urged food companies to label packages with a clear declaration to cook thoroughly after the discovery of HEV infection caused by Figatellu (Colson et al., 2010).

Many factors need to be fulfilled for successful transmission of HEV through consumption of pork. The meat has to be contaminated and then it has to be ingested raw or undercooked. In our study at least three pigs from a single batch had HEV in their internal organs at slaughter and prevalence's as high as 11% in commercial pig livers have previously been reported. Thus, the first criteria are fulfilled, because it is reasonable to conclude that HEV is present in unprocessed pork at relative high prevalence. However, raw liver or other pork organs are not a very popular commodity in Denmark (many stores don't sell it, and others only sell it once a week) thus the exposure would be limited. On the other hand, baked paté is extremely popular in Denmark. Insufficient heat treatment of these patés, where finely minced pork liver is the main ingredient, could expose a large part of the Danish population to HEV. In the study of Barnaud et al. (2012), 20 minutes at 71°C was necessary to completely inactivate HEV in a liver paté matrix. Under commercial production conditions, paté undergo a far more effective heat treatment so it is reasonable to assume that

Discussion

inactivation of HEV in general will occur in the food production line of these liver patés. This has not been documented, however, as standard food hygiene control programs do not include test for viral contamination. If the relative high seroprevalence in the population in countries like Denmark is due to exposure to HEV transmitted by insufficiently heat treated food products containing pork, clusters of cases or even large outbreaks would be expected as seen for other enteric food borne pathogens such as salmonella or Norovirus but this is very seldom seen for HEV that appear more sporadically. The key difference could be the viral load since the outcome of HEV infection appears to be dose dependent (Meng et al., 1998a). Mass production of food with pork components would lead to a considerable dilution of contaminating virus and further reduction in viral loads is expected even after insufficient heat treatments. Thus, combined these factors would contribute to a reduction in the number of infectious particles to a level that would induce only subclinical disease in exposed people. Furthermore, a possible low virulence in humans of the porcine adapted genotype 3 HEV could further explain why relative few clinical cases and no clusters of HEV outbreaks are seen even in populations with high seroprevalences.

Human HEV cases have mainly been regarded as a foodborne disease with consumption of animal meat and especially pork products as the main source of infection as discussed above. It is, however, reasonable to speculate that the high prevalence recorded in the human population is not entirely due to ingestion of un(der)cooked and HEV infected pork products. The transfer of HEV in the developing world is primarily by the fecal-oral route through contaminated drinking water. Intriguingly, the seroprevalences in these regions are still on the level of the seroprevalences recently reported in Europe. We showed that HEV indeed have potential to contaminate water reservoirs following application of animal manure to fields which are very common practice in Denmark. We did not find HEV in the groundwater screen, only RV, however RV leached through soil in a significantly different way than HEV, and the leaching pattern suggested that the sampling of groundwater was performed too late after application of slurry for the detection of HEV. Should HEV indeed leach into groundwater periodically during the slurry application seasons, it could greatly widen the number of people exposed to HEV originating in pigs, and thereby possibly contribute to the high seroprevalence. Furthermore, based on our study, the viral load would be very low in water samples and as HEV infection appears to be dose dependent (Meng et al., 1998a), waterborne HEV would still results in low number of clinical cases but accompanied by relatively high seroconversion rate due to the widespread exposure of contaminated water to humans. The fact that G1 and G2 are not endemic in the western world testifies to the higher sanitary level and the

lack of an endemic reservoir as seen with G3 and G4, which is the only genotypes found in pigs. G1 has, however, also been found in sewage in urban parts of Spain and in mussels in Italy (Donia et al., 2012)(Clemente-Casares et al., 2003). It is possible that G3 and G4 are less stable in the environment therefore not causing epidemics. G3 and G4 coexists with G1 in the eastern part of the world but still G1 or G2 were responsible for all well documented cases of waterborne epidemics. Instability of G3 and G4 would also explain negative results in shellfish although the number of samples tested in our study was low. Contamination of shellfish is tightly linked to the environmental contamination. We showed that PCV2, RV and HEV were drained from fields and purged into the water environment. In the shellfish, we only detected the extremely stable PCV2 virus. Stability of viruses is certainly a factor, as sampling time after contamination is crucial if one virus perishes after one month and the other persists for six months. The high seroprevalence recorded in Denmark is possibly a combination of many transmission routes. The work in this thesis strongly indicates that environmental factors might play an important role in the transmission of HEV in developed countries along with foodborne transmission.

10 Conclusion and perspectives

In this study a broader approach to studying HEV circulation in a developed country was taken, with focus on both environmental factors and on pig production practices. We detected HEV in the effluent of drain water from a field applied with slurry, indicating a potentially important environmental problem and an alternative route of transmission for HEV in developed countries. PCV2 and Rotavirus group A (RV-A) viruses were also detected in these effluents and RV-A was even present in groundwater, highlighting the importance of a better understanding of viral leaching in soil. The detection of PCV2 in drain water coincided with the detection in a high fraction of the analyzed shellfish samples. We propose the use of PCV2 as an indicator of porcine waste in mussels due to its seemingly high prevalence and known stability. This could be a beneficial tool to isolate the source of microbial pollution derived from porcine waste of water reservoirs. The high prevalence of HEV in pig herds further adds to the concern about the vast amount of pig slurry that is applied to fields each year. In our longitudinal study, we showed that a large fraction of the pigs shed virus even at the age of slaughter. By ensuring piglets receive MatAb's we also showed that the fraction of shedders can be significantly reduced. This in turn would also reduce the amount of HEV found in pig slurry. Furthermore, we showed that pigs shedding HEV at time of slaughter were relatively prevalent and that these positive pigs also were positive in various internal organs

such as liver and lungs despite the intensive measures taken to avoid fecal cross contamination at the process lines. The finding of a liver in a grocery store that contained more than a billion copies of HEV per gram were alarming and emphasized that Danish consumers are indeed in risk of contracting HEV through ingestion of uncooked pork. Furthermore, swine offal such as lungs are often used as animal feed, implying a potential risk for infection of e.g. mink through feed. We did, however, not find any swine associated HEV genotypes in mink, but did discover a novel variant of HEV closely related to the variant recently discovered in ferrets and rats.

Thus, based on three years of research on HEV, the perspective for transmission of HEV in developed world has been broadened and these new data further suggest that environmental control of viruses in general are urgently needed. Also potential ways to limit the HEV excretion in swine herds was paved, which offer important tools in the future control of this emerging zoonosis.

Perspectives: Combined with the knowledge that HEV is present in waste water taken from inlets to WWTP and the high viral load in sewage, our study on animal contribution of HEV to the water environment marks the first step in uncovering if HEV is waterborne in the western world. To gain a better understanding on the impact of slurry application on groundwater sterility, screening samples from municipal- or privately owned wells located near fields and taken in short intervals after slurry application, would give much needed information. Furthermore, infectivity of the positive samples found in this project is still questionable. With the development of more efficient cell lines, infectivity of environmental samples should be studied. The overall stability of HEV in the water environment based on G3 and G4 are also needed to evaluate the potential risk of HEV derived from pigs. Furthermore, the minimal infectious dose of HEV for an immune response (seroconversion) as well as for induction of clinical disease would be important factors and further studies should be performed using pigs and primates as models. The stability also translates into ingestion of pork products. Initial steps have been made to uncover the heat treatment necessary to inactivate HEV, however the large viral load in liver (we recorded more than 10^9 copies per gram of liver) requires high reduction rates just to reach the level of an infectious dose used in experimental trials. Further studies in different processed food matrices are needed to ensure a high level of food safety regarding HEV.

We report a perfect correlation between fecal sample and internal organ positivity for HEV. This study is however based on only 10 animals. A larger study is therefore needed to evaluate this

correlation. Preferably the samples should be taken from naturally infected animals e.g. at a slaughterhouse.

Mussels are excellent environmental bio-monitors provided that they accumulate the pathogen assayed. Shellfish have previously been used to evaluate the contamination of seawater with HEV (Donia et al., 2012). Our study showed that HEV was present in effluent from drained fields, the use of mussels as bio-markers in streams, which connect agricultural farm land with larger water reservoirs e.g. fjords and sea, could widen our understanding of the potential environmental circulation of HEV. There are a number of prevalence studies performed on mussels, however seasonal occurrence and the ability of mussels to accumulate and purge HEV is unknown. Further studies are needed in that regard to make well founded conclusions with respect to the role shellfish consumption plays in the transfer of HEV.

Only few of the detected HEV-like viruses are able to infect humans and only few of the viruses are real pathogens in the main hosts, but there is a complete lack of knowledge on the viral and host derived factors that determine if a given host is susceptible. Thus, research aiming at defining host and virulence markers is highly needed. These studies should include - but not be limited to - identifying the viral receptor(s). This in turn would facilitate culturing of the virus which could lead to development of better vaccines and by that offer better control measures in humans and in animals.

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12 Appendix I - Hepatitis E virus: En overset zoonose, der smitter fra svin

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Indsendt til: Dansk Veterinærtidsskrift

Summary

In recent years, Hepatitis E virus (HEV) has been reported as the etiological agent in multiple cases of human hepatitis also in Denmark. Pigs are considered to be the source of these sporadic HEV infections, but the exact epidemiological link between humans and pigs is unclear. The increased awareness on HEV has led to an increased number of reported cases of HEV induced hepatitis, however, HEV is probably still under diagnosed. The present paper present a basic overview on important features of Hepatitis E virus with special emphasize on the zoonotic aspects. Furthermore, results of research performed on this emerging pathogen in Denmark during recent years are described.

Resume

Hepatitis E virus (HEV) har de seneste år vist sig at være forbundet med flere tilfælde af leverbetændelse hos mennesker i Danmark. Svin menes at være smittekilde ved disse sporadiske tilfælde, men det er endnu ikke helt klarlagt hvordan virusset smitter fra dyr til mennesker. Øget opmærksomhed har ført til flere påvisninger af kliniske tilfælde af HEV betinget leverbetændelse, men den manglende information på området gør, at sygdommen formentligt stadig er underdiagnosticeret. I denne artikel præsenteres de vigtigste aspekter vedr. Hepatitis E virus med specielt fokus på de zoonotiske aspekter. Endvidere gives en beskrivelse af resultater af de danske forskningsaktiviteter, der er gennemført indenfor området de senere år

12.1.1 Fokus på HEV

Det er først nu - 30 år efter virusset blev isoleret - at der i den vestlige verden er begyndt at komme fokus på HEV's zoonotiske potentiale, og at dette virus kan udgøre et signifikant sundhedsproblem. Smitteveje for HEV i den vestlige verden er ikke afdækket, og selvom svin antages at være reservoir for human smitte af HEV, er det ikke endeligt dokumenteret, at der sker smitte fra svin til mennesker, eller om svin og mennesker smittes fra et fælles reservoir. Der er derfor et behov for at undersøge forskellige miljøer for tilstedeværelsen af HEV, før forebyggende tiltag kan iværksættes. Denne oversigtsartikel giver et summarisk overblik over de vigtigste resultater af disse undersøgelser med fokus på de zoonotiske aspekter.

12.1.2 Forårsager epidemier ved dårlig hygiejne

Hepatitis E virus (HEV) er et enkeltstretet RNA virus, der tilhører familien *Hepeviridae*. Efter opdagelsen af Hepatitis A og B, blev sygdommen fremkaldt af HEV kaldt enterisk non-A non-B hepatitis. Først i 1983 isolerede Balayan et al. HEV (1). Dette gjorde han ved at ekstrahere virus til en vandig fase fra fæces fra syge patienter, og Balayan selv indtog den virusholdige vandfase. Ca. 36 dage senere udviklede han akut hepatitis, der varede i tre uger, og han udskilte HEV 28 dage efter indtagelse og knap tre uger frem. HEV fik benævnelsen "E", da den er enterisk og forårsager epidemier (1). Retrospektive undersøgelser påviste senere, at HEV har været årsagen til omfattende hepatitisepidemier i Asien siden 1950. Den seneste epidemi blev rapporteret fra en flygtningelejr i Syd Sudan, Afrika i januar 2013. Årsagen til epidemierne er typisk dårlige sanitære forhold, der medfører smitte via fæcal-oral rute typisk via drikkevandet. Dette er baggrunden for, at det udelukkende er i udviklingslande man ser store epidemier. I Danmark og resten af den vestlige verden ses HEV typisk som sporadiske, kliniske tilfælde.

12.1.3 2 ud af 4 genotyper er zoonotiske

HEV kan inddeles i fire genotyper (G1 – G4), hvor G1 og G2 kun er påvist i mennesker, mens G3 og G4 forekomme hos flere dyrearter og betragtes som zoonoser (2). Alle fire genotyper tilhører den samme serotype, så det er ikke muligt via en serumprøve at undersøge, hvilken genotype der har forårsaget infektionen. Dette betyder formodentlig også, at antistoffer udviklet mod én genotype også beskytter mod de øvrige genotyper, hvilket er en fordel ved vaccineudvikling. Der er udviklet en vaccine til human brug, der er godkendt i Kina, men den er stadig ikke kommercielt tilgængelig andre steder (3). Ud over de fire primære genotyper er der flere artsspecifikke HEV-varianter (se figur 1). Kendskabet til disse er stadig meget begrænset, men den aviære variant (aHEV) forårsager problemer

i fjerkræproduktionen, hvor høns får forstørret lever og milt. Der er ikke rapporteret om smitte af mennesker med de artsspecifikke varianter.

12.1.4 HEV genotype 1 og 2 er problematiske for udsatte grupper

Genotype 1 og 2 er udelukkende fundet i mennesker og er endemisk forekommende i henholdsvis Asien/Afrika og Afrika/Mexico. Disse genotyper forårsager langt størstedelen af de globale humane tilfælde, da det er disse genotyper, der er årsagen til epidemier (4). Der er i flere tilfælde registreret G1 i danske humane sager, men patienter inficeret med denne genotype har oftest en rejseanamnese, der har kunnet forklare infektionen med denne genotype. Genotype 1 og 2 betragtes derfor ikke som endemiske i Europa eller Nordamerika. HEV infektionen præsenterer sig som en akut leverbetændelse, der i de fleste tilfælde går i sig selv, men kan udvikle sig til cirrose og leversvigt. De kliniske tegn er varierende grader af: gulsot, kvalme, udmattelse, diarre, feber, smerter i maveregionen og forstørrelse af leveren. Sunde og raske mennesker vil ofte opleve et forholdsvist mildt sygdomsforløb, hvor de kliniske tegn kan forveksles med f.eks. mild madforgiftning, eller have et helt subklinisk forløb (5). HEV udskilles fækalt i en periode på ca. to uger, men udskillelsen kan være ekstremt forlænget til over 50 dage (6). Den generelle dødelighed er under 4%, dog bliver der rapporteret en dødelighed på op mod 20% af gravide smittet med G1 og G2 (7). HEV rammer hele populationen, men der er en overvægt af mænd, der udvikler kliniske symptomer under et udbrud (8). Dødeligheden blandt patienter med eksisterende kroniske leversygdomme, der inficeres med HEV, kan nå 70% (9). Dette er baggrunden for at HEV også anses for at være særdeles problematisk for særligt udsatte grupper som transplantations patienter, kræftpatienter der undergår kemoterapi, samt andre med nedsat immunforsvar.

12.1.5 HEV Genotype 3 og 4 formodes at være mindre virulente

Genotype 3 findes globalt, mens genotype 4 først for nylig er fundet i Europa (10) og stadig ikke er rapporteret i Amerika. Denne genotype har ellers primært cirkuleret i Kina og Japan. Der er færre rapporter om fatale tilfælde, og der er heller ikke påvist dødelighed blandt gravide ved infektion med disse genotyper. Derfor opfattes G3 og G4 som mindre virulente end G1 og G2, men dette kan skyldes, at smitte med HEV G3 og G4 sker sporadisk og ikke som epidemier. Sygdomsforløbet ved disse sporadiske tilfælde er nemlig det samme som for G1 og G2. Derudover er G3 også fundet i transplantationspatienter, der har fået en kronisk infektion af HEV (11). I den vestlige verden står G3, og nu også G4, for alle de lokalt erhvervede tilfælde af HEV.

12.1.6 HEV giver ikke sygdom hos svin

G3 og G4 er fundet i svin i det meste af verden, og svin betragtes derfor som det primære reservoir for HEV. Svin inficeret med HEV udskiller, ligesom mennesker, viruspartikler i store mængder gennem fæces. Det høje antal viruspartikler bevirker, at der er et højt smittepres i staldene. I infektionsforsøg fik HEV-negative grise viræmi to uger efter kontakt med svin, der udskilte HEV (12). Man har også påvist den zoonotiske risiko ved at inficere svin med G3 isoleret fra mennesker, mens det omvendt ikke var muligt at inficere grisene med G1 og G2 (12). Ved infektion af grisene med G3 observerede man ingen kliniske tegn, hvilket også er tilfældet for naturligt inficerede svin i besætninger. Da hepatitis er et af de vigtige kliniske symptomer på Postweaning Multisystemic Wasting Syndrome (PMWS) har man undersøgt, om HEV spiller en rolle for udvikling af denne sygdom. Der blev ikke fundet en direkte sammenhæng, selvom HEV blev fundet i grise med PMWS. I samme undersøgelse så man milde læsioner på leveren forårsaget af HEV (13). Om HEV spiller en rolle i andre multifaktorielle sygdomme vides ikke.

12.1.7 Smågrise smittes i 8-12 ugers alderen

Smittedynamikken samt det kliniske sygdomsforløb i svin, er ikke endeligt klarlagt. Den gængse opfattelse er, at smågrisene er beskyttet af antistoffer fra moderdyret, og når disse falder til et ikke-beskyttende niveau, bliver den straks inficeret af HEV på grund af det høje smittepres i stalden. Infektion vil altså typisk ske omkring 8-12 ugers alderen. Herefter vil der være en periode med viræmi og udskillelse af HEV (14). Under det beskrevne forløb burde grisen være fri for virus inden slagting, men da der er påvist HEV i produkter (primært i leveren) fra svin i supermarkeder, må der være svin, der enten (gen)smittes senere eller har et længere infektionsforløb (15, 16).

12.1.8 HEV: En relativt ubeskrevet zoonose

På grund af det zoonotiske aspekt er der foretaget undersøgelser med henblik på at påvise, hvor HEV opformerer i grisen. Som forventet er leveren fundet positiv, men virusopformering ses også i tynd- og tyktarmen samt i lymfeknuder. På trods af at der ikke er fundet tegn på, at HEV skulle opformerer i musklerne på grisen (17), er der flere rapporter om fund af HEV i kød (18). Inkubationstiden af HEV varierer fra et par uger til et par måneder. Denne lange inkubationstid har gjort det svært at spore smitekilden i de fleste sygdomstilfælde, da denne ofte er spist eller bortskaffet, når sygdomstegn opstår. Dokumentation af, at der er sket en zoonotisk overførsel, er derfor vanskelig. Dog er der i Japan og Sydfrankrig i voldsomme akutte tilfælde med kort inkubationstid påvist identiske HEV-isolater i patienter og i kød. I Japan blev HEV fundet i nedlagt hjortekød og vildsvinekød. Årsagen viste sig at være utilstrækkelig varmebehandling af kødet før konsumering.

12.1.9 HEV i rå svineprodukter gav anledning til sygdom

I Sydfrankrig, hvor prævalensen af HEV er meget høj i mennesker, spises en særlig delikatesse kaldet ”figatelli”. Dette er en leverpølse, der traditionelt spises rå. I det dokumenterede zoonotiske tilfælde blev figatelli serveret for en familie på fem hvoraf tre familiemedlemmer spiste af leverpølsen og kun disse tre blev syge og diagnosticeret med HEV der var 100% identisk til det virus, der blev påvist i pølsen (19). Tilfælde med nedlagt vildt er også rapporteret i Europa, hvor tyske jægere havde nedlagt et vildsvin og tilberedt det utilstrækkeligt. Ud over vildsvin er der også påvist HEV G3 i rådyrbestanden i Ungarn (20). I Danmark er der ikke fundet så definitive beviser på zoonotiske overførelser. Der er dog lavet et fylogenetisk studie, hvor man sammenligner sekvenser af HEV-genomet isoleret fra mennesker med virus isoleret fra svin. Den fylogenetiske analyse viste at humane sekvenser og svinesekvenser grupperede sig med hinanden (figur 2), hvilket er en stærk indikation på, at det er de samme virusstammer, der findes i svin og mennesker. I figur 2 ses det også, at et humant tilfælde af HEV fra Danmark ligger tæt på et HEV-isolat fra en spansk gris (markeret med *). Det viste sig, at denne person havde været på ferie i Spanien (21). Disse undersøgelser antyder kraftigt, at svin og mennesker smittes af samme reservoir men ikke at smitten nødvendigvis sker fra svin til mennesker.

12.1.10 HEV i Danmark

I 2008 foretog PB Christensen et al. en undersøgelse af HEV antistoffer blandt danske bloddonorer. Undersøgelsen omfattede prøver fra både 1983 og 2003. I 1983 var 31,6 % serologiske positive, mens andele af positive var faldet til 20,6 % i 2003. Samtidig blev blodprøver fra landmænd analyseret, og det viste sig, at 50,3 % var positive (22). Der var altså signifikant flere landmænd, der var positive for antistoffer mod HEV, hvilket kunne antyde, at tæt kontakt til svin var en risikofaktor. Fra januar 2010 til marts 2013 blev der indsendt 1.255 prøver fra 919 patienter til Statens Seruminstitut for diagnostisk test for HEV. Prøver bliver indsendt pga. negativt resultat ved diagnostisk test for hepatitis A, B, C, og D, Human cytomegalovirus (CMV), Epstein-Barr virus (EBV), autoimmun hepatitis samt manglende rejseanamnese og ved kontakt med svin. I den samme periode er der identificeret en patient med kronisk HEV infektion efter levertransplantation.

Udbredelsen af HEV i de danske svinebesætninger blev undersøgt i 2009. Her blev det påvist, at mindst en ud af tre søer havde udviklet antistoffer mod HEV i 91,5 % af besætningerne (n=71), og mere end 50 % af de testede grise udskilte virus (23). Undersøgelser foretaget i et andet projekt påviste, at alle karakteriserede HEV-typer fra danske grise tilhørte genotype 3, hvilket også er den genotype, der indtil for nyligt var rapporteret i grise i resten af den vestlige verden (24).

12.1.11 HEV findes flere steder i miljøet

I udviklingslandene spiller miljøet og de sanitære forhold den største rolle for spredningen af HEV. Tidligere har der været en tendens til, at dette aspekt er blevet negligeret i Vesten. På grund af et stadig større behov for rent drikkevand samtidig med at tilgængeligheden af vand af drikkekvalitet er faldende, har der de senere år været stigende interesse for at undersøge forekomsten af patogener - herunder virus - i vand. I et spansk studie monitorerede man spildevandet i Barcelona over en længere periode samt undersøgte et begrænset antal spildevandsprøver fra Sverige, Grækenland, Frankrig og USA. Prøverne fra Barcelona blev ved med at være HEV-positive over en årrække, og der blev fundet positive prøver i Frankrig og USA (25). Disse resultater understregede, at HEV er endemisk forekommende også i de industrialiserede lande, men undersøgelserne påviste ikke smitekilden. Tilsvarende studier i Cairo har sandsynliggjort, at HEV påvist i spildevand er infektiøst, idet at 50% af personalet på et vand-rensningsanlæg havde antistoffer mod HEV. Til sammenligning havde kun 30% af kontrolgruppen antistoffer mod HEV (26). Denne gruppe havde samme profil, men arbejdede i et kontormiljø.

12.1.12 Naturligt reservoir – østers?

En udbredt indikator for mikrobiel forurening i vandmiljøer er forekomst af mikroorganismer i skaldyr. Dette skyldes, at skaldyr filtrerer store mængder vand dagligt, hvorved niveauet af mikroorganismer akkumuleres. I Danmark og i resten af Europa er der stærk kontrol med skaldyrproduktionen. Der er en løbende kontrol med bakterie- og toksinniveauerne, mens der ikke er en løbende kontrol med virus (27). Kilden for kontaminering af skaldyr er formodentlig udledning af spildevand samt overløb fra marker i forbindelse med udbringning af gylle, til de områder hvor der dyrkes skaldyr. I England lavede man en undersøgelse af muslinger dyrket ved kysten. Her var et højt antal prøver positive for HEV (92 % på vestkysten, 52 % på østkysten) (28). Det fremgår ikke af undersøgelsen, om prøverne blev taget fra områder godkendt til direkte konsum. Problemet understreges af at indtagelse af skaldyr ofte påvises som årsag til akut udbrud af virale sygdomme, da de i stort omfang konsumeres rå. De HEV-stammer, man har fundet i de europæiske skaldyr, er G3; der er identisk med den genotyper man finder i svin og mennesker.

12.1.13 Mere forskning i smitteveje

Både skaldyr og vand kan altså være reservoir for HEV som smittekilde, idet virus spredes fra grisefarmene gennem f.eks. gylle i naturen. Prøver fra patienter med leverbetændelse blev indtil for ganske nyligt ikke rutinemæssigt undersøgt for HEV i Danmark, men dette er nu ændret, hvilket er

et skridt i den rigtige retning. Forhåbentligt kan dette føre til mere forskning og dermed viden om smitteveje og – kilder, så vi får viden der kan anvendes til at kontrollere denne oversete zoonose.

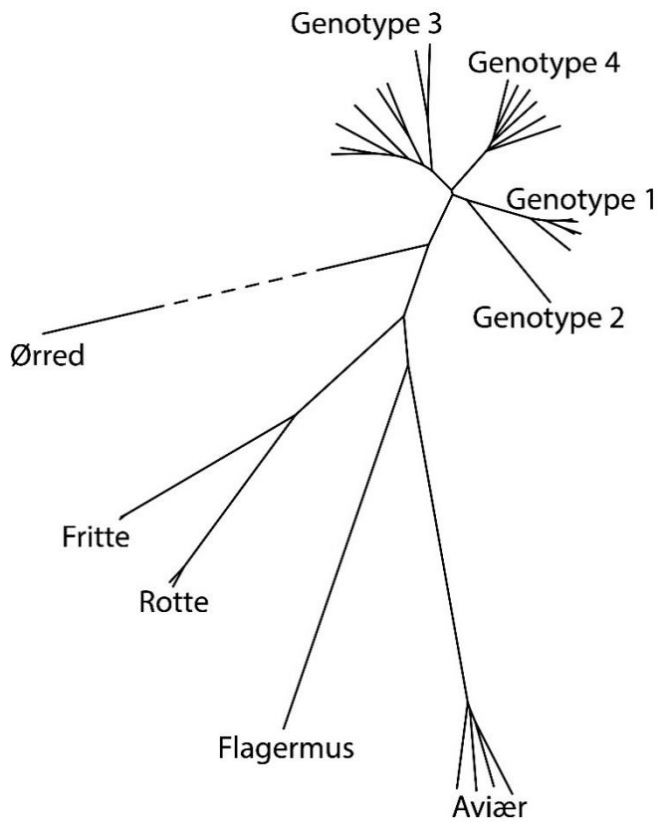
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Figur 1: Fylogenetisk træ baseret på fuld længde sekvenser af genomet fra HEV isoleret fra forskellige værter.



Figur 2: Fylogenetisk træ med HEV isoleret fra mennesker og svin. Adapteret fra Norder et al.

