Hepatitis E virus in the virome of water and animals

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Cover illustration: Hao Wang

To my beloved parents

"The water that bears the boat is the same that swallows it"
Xun Kuang

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ABSTRACT

This thesis was aimed to investigate viruses in different animals and water to get some understanding of viruses that disseminate into the environment. Next generation sequencing (NGS) was used to explore the virome from raw to treated water at two Swedish drinking water treatment plants (DWTP) and in tap water. The amount of viruses was lowered with 3-4 log10 after the treatments. The viral diversity was reduced from 26 different virus families in raw water to 12 in tap water. Hepatitis E virus (HEV), subtypes HEV3c/i and HEV3a, were identified in most water samples, with 10-130 International Units of HEV RNA/mL tap water. The viral diversity was also investigated in incoming and treated wastewater at two Swedish wastewater treatment plants (WWTP) in Knivsta, Stockholm, and Gryaab in Gothenburg. Ozone treatment was used after conventional treatment before the release of the treated wastewater from Knivsta WWTP. At least 327 virus species, belonging to 25 known virus families were detected in the raw wastewater. The virus concentration was reduced by 1-6 log10 for 21 human related viruses, with lowest removal efficiency for adenovirus. At Gryaab WWTP, seasonal differences in presence and concentration of 13 human viruses in raw and treated wastewater were investigated during one year. Twelve of the viruses were detected throughout the year in influent and effluent wastewater by either qPCR or NGS. HEV was found in effluents when released into the Göta River. The concentrations of all viruses in influent were reduced by 3-4 log10 in the effluents. Since HEV was identified in most water samples, its prevalence among their major hosts, wild boars and pigs, was investigated. HEV in Spanish and Swedish wild boars were compared. HEV RNA was found in 20% in Spanish wild boars vs. 15% in Swedish wild boars, while anti-HEV was significantly higher among Spanish wild boars (59% vs. 8%). Most Swedish and some Spanish wild boars were infected by subtype HEV3f, while several Spanish wild boars were infected by divergent HEV3c/i strains, indicating regional differences in infecting HEV strains. The Swedish wild boar strains were similar to strains from infected Swedes and Swedish domestic pigs. These wild boars were also infected with at least 27 different viruses, identified by NGS on liver samples. HEV3 was identified in 22% of piglets from 77% of 30 investigated pig farms sampled twice with more than one year apart. Most piglets were infected with HEV3f or HEV3e. Each pig farm had a unique HEV strain, and several strains were similar to human HEV3 strains.

These studies showed that viruses are disseminated into the environment both from raw water, treated wastewater and animals, and may be found in tap water. The HEV3 strains identified in drinking water were different from those isolated from Swedish pigs and wild boars, and similar to strains from humans with unknown source of infection, indicating waterborne transmission also for HEV3.

SAMMANFATTNING PÅ SVENSKA

Många olika virus kan spridas via djur eller vatten till människa och andra djur. För att undersöka variabiliteten av virus i olika vattenprov utvecklade vi en metod som kan koncentrera virus från större mängder av vatten (>1000 L). Virus påvisades genetiskt antingen med qPCR eller next generation sequencing (NGS).

Virusförekomst undersöktes i avloppsvatten före och efter rening från ett reningsverk i Knivsta utanför Stockholm under tre veckor. Under försöksperioden hade en ozonanläggning kopplats till detta verk. Man avsåg att undersöka om ozonbehandlingen av det renade avloppsvattnet kunde minska mängden läkemedel, andra kemikalier och virus från vattnet innan det släpptes ut till en närliggande å. I det inkommande avloppsvattnet kunde minst 327 olika virustyper från 25 olika virusfamiljer identifieras, bl.a. hepatit E virus (HEV). Efter konventionell rening minskades antalet av de flesta virus 10 000 gånger och ozonbehandlingen kunde minska antalet ytterligare upp till 100 gånger. Vissa virus kunde ej längre påvisas redan efter konventionell rening, medan andra som adenovirus reducerades i betydligt lägre grad, även efter ozonbehandling. Detta visade att många virus kan avlägsnas från avloppsvatten vid konventionell rening, och ozon kunde eliminera ytterligare virus. Men fortfarande passerade ett antal virus reningen och följde med vattnet ut från reningsverket.

Denna studie följdes upp genom att undersöka virus i avloppsvatten före och efter rening under ett år i Ryaabs reningsverk i Göteborg. Prover från inkommande avloppsvatten togs varannan vecka, och större mängder utgående renat avloppsvatten undersöktes varje månad. Mängden av 13 virus som ger gastroenterit bestämdes som i föregående studie med qPCR. Om man antar att en smittad person utsöndrar 10^{11} virus per vecka, kunde antalet smittade som utsöndrat virus till avloppsvattnet beräknas. Detta antal relaterades till antalet patienter med påvisad virusförekomst hos boende i reningsverkets upptagningsområde. Elva av 13 undersökta virus kunde påvisas i de 26 avloppsvattenproven. Koncentrationen av virus varierade med tid för olika virus, för vissa virus var den högst under de kalla månaderna, som för norovirus GII. Andra virus hade en jämn koncentration över året, som aichi virus och parechovirus. Samtliga 11 virustyper som identifierats i inkommande avloppsvatten återfanns även i det renade vattnet. Koncentrationen av virus i omkring 11 m^3 av det renade vattnet hade reducerats 1000 till 10000 gånger innan det släpptes ut till Götaälv. Trots detta var det 20 till 200 000 virus partiklar per L av det utgående vattnet. När virus förekomst i det utgående vattnet även undersöktes med NGS påvisades större koncentrationer av bakteriofager, växtvirus, och även humana virus som HEV. Dessa HEV stammar visades dels tillhöra subtyp HEV3c/i som ofta isoleras från svenska kroniska bärare av HEV, samt HEV från råtta. Dessa var genetisk skilda från de HEV stammar isolerade från råttor i Europa och USA.

I en studie jämfördes virusförekomsten i prov från två vattenreningsverk i Göteborg. Det ena reningsverket använder 20nm ultrafilter (UF) det andra UV ljus som det sista steget i reningsproceduren av dricksvattnet. Prover togs innan, under och efter reningen, samt under tre följande nätter i kranvatten i Göteborg. Stora mängder virus tillhörande 26 olika virusfamiljer kunde identifieras i råvatten innan rening. Efter de olika reningsstegen visades

UF reducera mängden virus 10 000 gånger, medan UV reducerade mängden 1000 gånger. Trots detta påvisades virus tillhörande 12-18 olika familjer i kranvattnen. Bland dessa virus fanns HEV och samma bakteriofager och växtvirus som identifierades i de renade avloppsvattnen. HEV stammarna från kranvatten visades vara subtyp HEV3c/i och genetisk likna de HEV stammar som fanns i vattnen efter UF innan det släpptes ut till vattenledningarna och i renat avloppsvatten från Gryaabs reningsverk i Göteborg. Detta pekar på att HEV3, bl.a. HEV3c/i kan spridas via vatten.

Då HEV påvisats i flera olika vatten, undersöktes förekomst av detta virus hos svenska vildsvin och spanska vildsvin i Barcelona. Denna studie undersökte dels prevalens av HEV och dels om de virus som infekterat vildsvin också kunde påvisas hos människa. Antikroppar mot HEV fanns hos 59% av de spanska vildsvinen, medan de endast kunde påvisas hos 8% av de svenska vildsvinen. Däremot var förekomsten av smittade djur ungefär densamma, där 20% av spanska och 15% av de svenska vildsvinen med påvisbart HEV RNA i blod och/eller feces. Då de spanska vildsvinen var äldre skulle detta kunna förklara skillnaderna i antikroppsförekomst. Men detta förklarar inte varför förekomsten av HEV RNA var lika stor. De flesta svenska men bara några spanska vildsvin var smittade med subtyp HEV3f. De flesta spanska vildsvinen var smittade med en variant av HEV3c/i, vilket är den subtyp som ofta isoleras från kroniska bärare av HEV i många europeiska länder inklusive Sverige. Detta skulle kunna visa på att de spanska vildsvinen oftare var kroniska bärare än de svenska. Vissa stammar från de svenska vildsvinen liknade stammar från infekterade människor. Leverprov från de svenska vildsvinen visade att djuren var smittade med minst 27 andra virus. Vissa av dessa skulle kunna spridas till tamgrisar, som porcine astrovirus, sapovirus, boca virus samt picornavirus.

Förekomst av HEV RNA undersöktes även hos griskultingar i 30 svenska grisfarmar som följdes upp med två års mellanrum. 77% av gårdarna hade smittade kultingar och 20% av alla undersökta kultingar var smittade. När HEV stammarna typades visades att varje gård hade sin egna unika HEV stam som fanns kvar på gården under minst två år. Detta var oberoende av gårdstyp, om den var sluten eller ekologisk där grisarna kan vistas utomhus. HEV stammarna tillhörde subtyperna HEV3e eller HEV3f. En gård hade en HEV3f stam som liknade stammar från svenska vildsvin från samma geografiska område. Andra gårdar hade stammar som liknade de som isolerats från svenskar med akut hepatit E. Detta pekar på att zoonotisk HEV smitta är vanlig i Sverige men även att vildsvin och tamgrisar kan smitta varandra.

Sammantaget har dessa studier visat att det är effektiva reningar från virus av såväl avloppsvatten som dricksvatten i Sverige. Trots denna effektiva rening finns det virus i badsjöar och även i kranvatten. En bakteriofag, gokushovirus, och ett växtvirus pepper mild mottle virus, som infekterar paprika, identifierades i hög frekvens och koncentration i samtliga vatten, och borde föreslås som markörer för effektiviteten av vattenreningen från virus. Även HEV3 återfanns frekvent i alla undersökta vatten. Detta virus visades infektera såväl grisar som vildsvin i hög frekvens. De stammar som isolerats från ett flertal smittade svenskar liknar de från såväl grisar som vildsvin. Detta tyder på att zoonotisk överföring förekommer frekvent i Sverige. Resultaten pekar även på att det är skillnader mellan olika subtyper av HEV3. Resultaten kan peka på att HEV3c/i orsakar oftare kronisk HEV infektion inte bara hos människa utan även hos vildsvin, och att HEV3 möjligen även är en vattenburen smitta.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. **Hao Wang**, Raquel Castillo-Contreras, Fredy Saguti, Jorge R López-Olvera, Marie Karlsson, Gregorio Mentaberre, Magnus Lindh, Jordi Serra-Cobo, Heléne Norder, Genetically similar hepatitis E virus strains infect both humans and wild boars in the Barcelona area, Spain, and Sweden. Transboundary and Emerging Diseases, 2019, 66, (2), 978-985.

II. **Hao Wang**, Marie Karlsson, Maria Lindberg, Kristina Nyström, Heléne Norder, Hepatitis E virus strains infecting Swedish domestic pigs are unique for each pig farm and remain in the farm for at least 2 years. Transboundary and Emerging Diseases, 2019, 66, (3), 1314-1323.

III. **Hao Wang**, Per Sikora, Carolin Rutgersson, Magnus Lindh, Tomas Brodin, Berndt Björlenius, D.G. Joakim Larsson, Heléne Norder, Differential removal of human pathogenic viruses from sewage by conventional and ozone treatments. International Journal of Hygiene and Environmental Health, 2018, 221, (3), 479-488.

IV. **Hao Wang**, Inger Kjellberg, Per Sikora, Henrik Rydberg, Magnus Lindh, Olof Bergstedt, Heléne Norder, Hepatitis E virus genotype 3 strains and a plethora of other viruses detected in raw and still in tap water. Water Research, 2019, 168, 115141.

V. **Hao Wang**, Julianna Neyvaldt, Lucica Enache, Per Sikora, Ann Mattsson, Anette Johansson, Magnus Lindh, Olof Bergstedt, Heléne Norder, One year seasonal variations of enteric viruses in incoming and treated water at a wastewater plant. Manuscript.

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ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic Acid
ORF	Open reading frame
ssDNA	Single-stranded DNA
IgM	Immunoglobulin M
IgG	Immunoglobulin G
ICTV	International Committee on the Taxonomy of Viruses
HEV	Hepatitis E virus
Met	Methyltransferase
Hel	Helicase
RdRp	RNA-dependent RNA polymerase
HAV	Hepatitis A virus
HAdV	Human adenovirus
HuNoV	Human norovirus
HAtV	Human astrovirus
HPeV	Human parechovirus
EV	Enterovirus
ELISA	Enzyme-linked immunosorbent assay
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription PCR
NGS	Next generation sequencing
WHO	World Health Organization
FIB	Fecal indicator bacteria
PMMoV	Pepper mild mottle virus
WWTP	Wastewater treatment plant
DWTP	Drinking water treatment plant
CML	Clinical Microbiology Laboratory
UV	Ultraviolet

UF	Ultrafiltration
PRRSV	Porcine reproductive and respiratory syndrome virus
ASFV	African swine fever virus
CSFV	Classical swine fever virus
qPCR	Quantitative polymerase chain reaction
OD	Optical density
IU	International Units
CPE	Cytopathic effect
UPGMA	Unweighted pair-group method using arithmetic averages
NJ	Neighbor-joining
BLAST	Basic local alignment search tool
NCBI	National Center for Biotechnology Information
PEG	Polyethylene glycol
HPV	Human papillomavirus
ICC	Integrated cell culture

1. INTRODUCTION

1.1 Hepatitis E virus

Hepatitis E virus (HEV) is a small, non-enveloped virus with a size of 27-34 nm. HEV was first described from an epidemic of non-A, non-B hepatitis from Kashmir in 1978 [1]. Later it was reported again in Afghanistan in 1980s after an outbreak of unexplained hepatitis in a Russian military camp [2]. Since then, the infections and outbreaks caused by HEV have been reported worldwide. HEV is one of the causative agents of acute and chronic viral hepatitis. According to World Health Organization (WHO), there are yearly about 20 million HEV infections worldwide, leading to an estimated 3.3 million symptomatic cases of hepatitis E [3]. In 2015, WHO estimated that hepatitis E infections had a mortality of 3.3% (approximately 44,000 deaths) [4]. HEV is mainly transmitted via the fecal-oral route, often by contaminated drinking water, but other routes of transmission, such as through ingestion of contaminated pork products, transfusion of infected blood products, and vertical transmission from mother to child, have also been identified.

1.1.1 The structure of HEV genome

HEV has a positive-sense, single-stranded RNA genome, approximately 7.2 kb in length. The virus genome contains three partially overlapping open reading frames (ORF1, ORF2, and ORF3), and short non-coding regions capped at the 5' terminus and polyadenylated at the 3' terminus [5]. ORF1 is the largest ORF and encodes for non-structural proteins involved in virus replication and protein processing. These proteins include methyltransferase (Met), helicase (Hel), and RNA-dependent RNA polymerase (RdRp; Figure 1). The function of the Met is to catalyse the capping of the virus RNA, while Hel and RdRp are needed to replicate the HEV RNA [6].
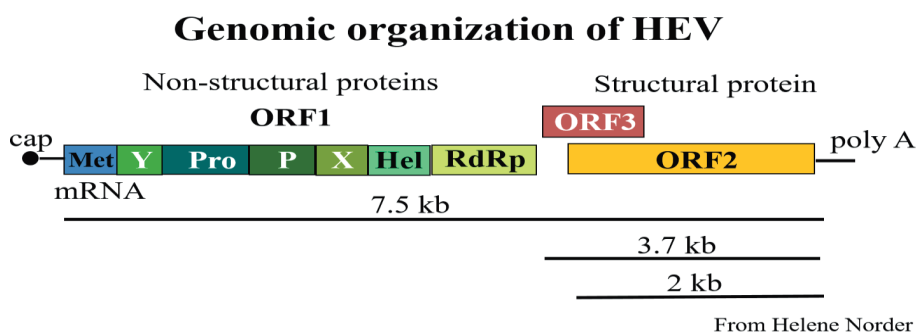
Figure 1. Genomic organization of HEV showing three open reading frames (ORF).

The ORF2 encodes the viral capsid protein of 660 amino acids. This protein assembles in to a capsid encompassing the virus RNA, and has epitopes for the virus to bind to the host cell. Neutralizing antibodies are directed to epitopes of ORF2. ORF3 overlaps with ORF2. The protein encoded by ORF3 is a small protein of 114 amino acids involved in virion morphogenesis and release [7, 8]. Apart from three ORFs, the RNA forms a stem-loop, the so called junction region, between ORF1 and ORF2/3. This structure is important for virus replication [9].

1.1.2 HEV taxonomy and global distribution

HEV was first classified to the *Caliciviridae* family based on its morphological properties. Subsequent sequence analysis showed that HEV sequences are distinct from *Caliciviridae*. HEV is therefore classified as a separate family, *Hepeviridae*, by the International Committee on the Taxonomy of Viruses (ICTV) [10]. There are two genera in the *Hepeviridae* family, *Piscihepevirus* and *Orthohepevirus*. The members belonging to the former genus infect trout (trout HEV), while the members in the latter genus infect mammals and birds (mammalian and avian HEV). The *Orthohepevirus* genus is further classified into four different species, as *Orthohepevirus* A, B, C, D [10]. HEV strains, which infect mammals, such as humans, pigs, wild boars, and camels, belong to *Orthohepevirus* A. Members from *Orthohepevirus* B include avian hepatitis E virus strains detected in chickens and wild birds [11, 12]. Strains from *Orthohepevirus* C have been detected in rats, ferrets, and mink [13-15]. *Orthohepevirus* D includes bat HEV and has a global distribution, but there is no evidence of its transmission to humans [16].

Table 1. Global distribution of HEV genotypes infecting humans.

Genotype	Host	Geographic Distribution	Zoonotic Potential
HEV1	human	Asia, Africa, Latin America	No
HEV2	human	Africa, Mexico	No
HEV3	human, pig, wild boar, deer, rabbit, etc.	Worldwide	Yes
HEV4	human, pig, wild boar, deer, goat, cow, etc.	Asia, central and western Europe	Yes
HEV7	human, camel	Middle East	Yes

HEV strains infecting humans belong to five of the eight genotypes forming *Orthohepevirus* A, HEV1-HEV4, and HEV7 (Table 1). Viruses belonging to each genotype have specific host ranges and geographical distribution. HEV1 and HEV2 can only infect humans and are mainly transmitted via contaminated water [17]. HEV1 has been isolated from large outbreaks and

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sporadic cases of hepatitis E in Asia, Africa and Latin America [18-20], where the disease is hyperendemic. HEV1 strains have also been isolated from cases in industrialized countries. Those infections were mostly associated with a travelling history to endemic areas [21]. HEV2 was first reported from Mexico [22]. Since then it has been isolated from outbreaks in some African countries [23, 24]. HEV3 can infect humans as well as several mammalian species, such as pig, wild boar, deer, and rabbit [25-28], and has a worldwide distribution. This genotype is responsible for sporadic cases of autochthonous hepatitis E in both developing and developed countries. HEV4, like HEV3, is zoonotic, and can also infect humans and animals, but with a limited geographical distribution. HEV4 infections were mainly reported from Asian countries, such as China and Japan [29, 30]. This genotype was also recently isolated in central and western Europe [31, 32]. HEV7 was first reported from dromedaries sampled in the United Arab Emirates in 2013 [33]. A patient from the same area who developed chronic hepatitis after liver transplantation was infected with HEV7. The route of infection was probably through consumption of camel-derived food products [34]. Based on the phylogenetic analysis, these genotypes can be further classified into different subtypes. For HEV1 and HEV2, six (1a-1f) and two (2a-2b) subtypes have been identified. HEV3 and HEV4 are more diverse, and are classified into eleven HEV3 (3a-3j, and 3ra) and nine HEV4 (4a-4i) subtypes [35]. Although several HEV genotypes have been identified, it is assumed that there is only one serotype.

1.1.3   HEV transmission

HEV is primarily transmitted via the fecal-oral route. In developing countries with poor sanitation, HEV infections are commonly waterborne transmitted. This is due to inadequate disposal and treatment of wastewater, which may contaminate the drinking and irrigation water. Contaminated water has caused large outbreaks of HEV with numerous cases [36-39]. All outbreaks described so far were caused by HEV1 and HEV2. HEV strains have been detected in raw wastewater from both developing and developed countries. These strains were closely related to strains circulating in the local populations and animals [40-42]. Surface water can be contaminated by fecal-shed HEV, and irrigation of fruits and vegetables using contaminated surface water may cause a public health hazard for HEV infection [43, 44]. In addition, several reports have shown that HEV infections were associated with consumption of shellfish, such as mussels and oysters, that have been growing in river or coastal water contaminated by HEV in several European countries [45, 46].

In developed countries, clean drinking water is easily accessed and the spread of HEV through water is controlled. The spread of HEV in these countries is mainly through foodborne zoonotic transmission. HEV3 and HEV4 strains have been detected in various tissues and organs of mammalian animals, such as domestic pigs, wild boars, deer, and rabbits [47]. The consumption of undercooked or raw tissues or organs, such as meat, liver sausage, and intestines, from HEV infected animals has been linked to clinical cases [48-51]. Contact exposures to the animal reservoirs, as domestic pigs and wild boars, are also considered source of HEV infection. Studies have shown a higher seroprevalence against HEV in hunters, swine farmers, slaughterhouse workers, and swine veterinarians, who have a frequent contact with HEV animal reservoirs, compared to the other populations [52-54].

Blood-borne transmission via blood transfusion or transplantation of solid organs have been reported in recent years. Most HEV infections are asymptomatic, but viremia can last for several weeks [55]. An asymptomatic HEV carrier may donate blood. The use of HEV viremic blood products could lead to acute HEV infection, even chronic hepatitis and cirrhosis, in the recipients [56-58]. This route of infection could cause severe hepatitis especially in transplant recipients and other immunocompromised individuals, such as HIV infected patients [59]. Vertical transmission from mother to fetus or child have also been described [60, 61], and intrauterine transmission of HEV is associated with maternal death or spontaneous abortion [62].

1.1.4   Symptoms of HEV infection

HEV causes diseases varying from subclinical to fulminant hepatitis. Most HEV infections are asymptomatic or mild without jaundice, while 5-30% of HEV infected individuals have classical clinical symptoms of hepatitis, as anorexia, myalgia, fever, nausea, vomiting, and jaundice. In rare cases, acute hepatitis E can develop into fulminant hepatitis, with a life-threatening risk for these patients [63]. Fulminant hepatitis may occur in infected pregnant women during the third trimester with a mortality rate between 15% and 30% [64]. In immunocompromised patients, such as organ transplant recipients and HIV-infected patients, the course of disease may progress to chronic hepatitis and cirrhosis, and their HEV replication and virus shedding may persist for a longer period [58, 65, 66].

The manifestations of a hepatitis E infection are dependent on genotype. HEV1 and HEV2 strains are restricted to humans. Infections with these genotypes vary from asymptomatic or mild illness to acute hepatitis and fulminant liver failure. Pregnant women infected with HEV1 and HEV2 are at risk of developing acute liver failure and have a high mortality [64, 67]. HEV3 and HEV4 infections mostly have a clinically silent course, with about 30% of the infected developing symptomatic acute hepatitis [68]. However, the reported chronic or persistent hepatitis E infections in immunosuppressed patients are all caused by HEV3 and HEV4, and are not observed in HEV1 and HEV2 infections. One study showed that HEV4 infections are associated with higher level of liver disease and more often lead to a more severe hepatitis than HEV3 infections [69].

HEV infections are also associated with several extrahepatic manifestations, especially neurological and renal manifestations. For neurological manifestations, most of the reported symptoms are Guillain-Barré syndrome, neuralgic amyotrophy, encephalitis, and myelitis [70]. Impaired renal functions, such as membranoproliferative and membranous glomerulonephritis, have been observed in both acute and chronic HEV patients [71, 72], and these patients were infected with HEV1 or HEV3. Other manifestations, such as acute pancreatitis, arthritis, and myocarditis, have also been documented. The evidences supporting these associations are not strong, since only a few cases have been reported [63]. Further studies are needed to confirm these associations.

1.1.5   HEV diagnosis

The incubation period for HEV infections usually ranges from 2 to 10 weeks with a mean of 25–50 days [73]. HEV RNA can be detected in the blood and feces during the incubation period and lasts for another 4-6 weeks. Meanwhile, the capsid antigen can persist in the blood for the same duration (Figure 2) [63, 74]. Anti-HEV IgM is a marker of recent or current HEV infection. Around 90% of the infected patients have produced detectable levels of this antibody at 2 weeks after infection, and the response lasts for up to 5 months or even longer [75]. The anti-HEV IgG response can be delayed when compared with IgM, but its antibody titers continue to rise to a higher level and may persist for several years. The exact duration of the IgG response remains uncertain.

HEV infection can be diagnosed either indirectly by detection of anti-HEV antibodies in blood or directly by detection of HEV RNA or capsid antigen in blood or feces. The enzyme-linked immunosorbent assay (ELISA) is used for the detection of anti-HEV antibodies (IgM and IgG), but all assays vary in performance. Analysis for anti-HEV IgM is recommended as the first-line diagnostic assay for acute HEV infection. For immunocompetent patients, high levels of anti-HEV IgM indicate acute infection in a routine clinical setting. For immunocompromised patients, the immune responses can be impaired, and for immunocompetent patients the IgM response may come late. Therefore, analysis for HEV RNA should complement the diagnosis [76, 77]. Anti-HEV IgG may persist for several years. Its detection is applied for seroprevalence studies, and for determining effectiveness of HEV vaccine [76]. The anti-HEV IgG and IgM assays have varying sensitivity and specificity for different genotypes, and cross reactions with other viral agents, which should be taken into consideration when diagnosing HEV infections [6].
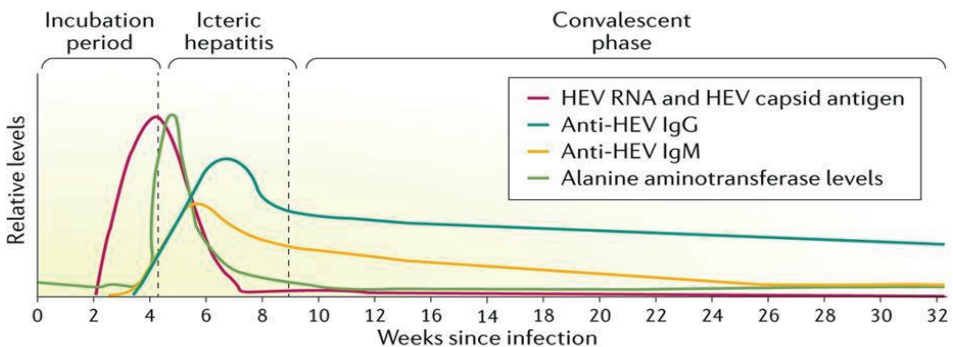

Figure 2. Schematic representation of hepatitis E virus infection. Cited from reference 63 with permission.

HEV RNA detection and quantification in blood, feces, or other body fluids using RNA amplification methods, such as RT-PCR and RT-qPCR, are regarded as the gold standard for detection of an active HEV infection. Analysis for HEV RNA can be extended for blood donor

screening, but this is still on debate in Sweden. Some European countries, like Germany, Ireland, United Kingdom, and the Netherlands, have introduced a nationwide blood screening. France has introduced selective screening of products to be donated to immunocompromised patients, while other countries are still evaluating the situation [78, 79]. One benefit of RNA amplification based detection of HEV is that sequences could be obtained and used for HEV genotyping. Knowledge of the genetic fingerprint of the strain may be used to identify the source of infection or to detect mutations associated with outcomes from antiviral therapy [80].

1.1.6   HEV treatment and vaccination

Currently, there is no specific treatment to alter the course of acute hepatitis E infection. In most immunocompetent individuals, the infection is spontaneously cleared without severe symptoms, and hospitalization is generally not required. However, severe acute hepatitis and fulminant hepatitis may occur. Hospitalization with supportive care and antiviral treatment are then required to avoid the development of acute liver failure or death [81, 82]. Ribavirin monotherapy is commonly used in the treatment of severe acute hepatitis E. This treatment has shown a prompt viral clearance and improved liver function [81-83]. For high risk groups, as pregnant women with HEV infection, hospitalization and antiviral treatment should also be considered. Despite ribavirin being a teratogenic drug [84], the lethal risk of untreated HEV to the mothers and their fetus is high, and antiviral therapy may be beneficial to them.

For immunosuppressed individuals with chronic hepatitis E, the infection may develop into cirrhosis if no treatment is given [85]. A reduction of the immunosuppressive therapy, especially of drugs targeting T cells, is the first step to be taken to clear the HEV infection [86]. This has been shown effective for one third of the patients [86, 87]. It is suggested that all immunosuppressed individuals should be screened for HEV for a rapid diagnosis, which may abate the risk for the patients to develop progressive liver disease [88]. Ribavirin has been shown to be an effective antiviral drug against hepatitis E also in immunocompromised patients [89, 90]. PEGylated IFNα has also been used successfully in a few liver transplant recipients [91, 92], but it could stimulate the immune system and increase the risk of transplant rejection. It is therefore recommended that IFNα therapy is applied only to those who do not respond to ribavirin [63].

An effective recombinant HEV subunit vaccine, HEV 239, has been licensed and used in China since 2011, but not in other parts of the world. Clinical trial showed that three doses of the vaccine was well tolerated and effective in the prevention of hepatitis E in the general Chinese population aged 16-65 years [68]. In 2014, WHO reviewed the HEV 239 vaccine and recommended a further trial and data on its safety in children, elderly, pregnant women, and other vulnerable populations [93]. WHO does not recommend routine use of the vaccine in national wide vaccination programmes. They suggest the use should be considered in special situations, as during outbreaks when the risk of hepatitis E and its complications is high, or with high mortality among infected pregnant females [4].

1.2   Virome in different types of water

1.2.1   Virome in wastewater

Wastewater, also known as sewage, has a complex composition. It mainly comes from the household wastewater, and enters into the wastewater treatment plant (WWTP) together with industrial wastewater, and rain and storm water. Since wastewater is a mixture from different sources, it contains plenty of organic and inorganic pollutants, microorganisms, and pharmaceutical pollutants. Among them, the microorganisms can be further divided into viruses, bacteria, protozoa, and parasites. Some of them are human pathogens, and could lead to human diseases. Wastewater is a critical component of the water management cycle. Therefore, effective treatment of the wastewater is needed before discharging it into the receiving water body considering of the risk it may cause for the public.

1.2.1.1 Wastewater treatment plant (WWTP)

In many developing countries, the release of untreated wastewater into the environment remains common due to the lack of treatment infrastructures, techniques, and financing, which causes a widespread water pollution. This situation may become worse with the progression of rapid urbanization, industrialization, population growth, and water resources depletion. The World Water Development Report from the United Nation showed that high-income countries treat about 70% of the sewage they generate, that ratio drops to 38% in upper middle-income countries and to 28% in lower middle-income countries. Only 8% undergo treatment in low-income countries. Overall, over 80% of all wastewater is discharged without treatment globally [94].

In developed countries, higher percentage of sewage is treated in WWTP before its release. In most conventional western WWTPs, the raw wastewater is treated with combined mechanical, biological, and chemical processes [95]. The treatments start with some form of mechanical treatment, including the use of a screen, grit chamber and primary sedimentation. During this stage, larger debris, such as stones, wood, paper, textiles, and plastics are separated and removed. Thereafter biological treatment is applied by using microorganisms, also known as active sludge process, during which chemical components, like phosphorous, nitrogen, and organic matters, are removed. Then phosphorous is precipitated by the addition of chemicals, such as aluminium and iron. Some WWTPs have strict requirements, a filtration of the treated water is performed to further separate sludge and particles. After the whole treatment processes, the treated wastewater is discharged into receiving waters, which usually are rivers, lakes or sea. The sludge produced during the treatment is collected and undergoes a separate sludge treatment. Thereafter the treated sludge can be used for other purposes, such as biogas or fertilizers.

WWTPs are normally not designed to remove substances as pharmaceutical residues and human diseases related pathogens. Recently, the reclaimed wastewater has been used as irrigation water in many countries, as in China, Indian, Pakistan, and Australia [96], where there is an increasing demand on water resources. However, the release of water with inadequate treatment or removal of pharmaceutical residues and pathogenic microorganisms may cause public health problems [97-99]. To further reduce these substances, advanced treatment, as UV irritation and ozone, is applied after conventional treatment to produce high quality reclaimed wastewater.

In Sweden, the entire both urban and industrial wastewater was directly discharged into lakes, rivers, and coastal areas until the sewage system was built in larger cities around late 19th century. The first was built in Gothenburg in 1866, followed by one in Stockholm in 1868. After that, the wastewater treatment system was developed slowly and it was far from sufficient, which led to a severe pollution of the water, especially near larger urban areas. Since the 1970s, the Swedish government started to invest in municipal wastewater treatment capacity, which significantly reduced the water pollution and improved the water quality [95]. Nowadays, almost all households in urban areas are connected to wastewater treatment systems. More than 95% of the wastewater is treated with conventional treatment methods. In addition, many larger industrial and mining facilities have their own WWTPs [100]. But there are still around a million households and a number of properties, such as summer houses, that are not connected to municipal wastewater services. They use small scale on-site treatments that often do not meet legal requirements [101].

1.2.1.2 Human pathogenic viruses in wastewater

Wastewater is a mixture of diverse sets of microorganisms, including human pathogenic viruses. These viruses are mostly enteric viruses, which are shed from feces, urine, and respiratory secretions of infected hosts (humans and animals), and then enter into wastewater. These enteric viruses belong to different virus families, but all are mainly transmitted by the fecal-oral route. They can persist for a long period in water environments [102-104]. Most reported common enteric viruses found in wastewater worldwide are hepatitis A virus (HAV), HEV, adenovirus, norovirus, rotavirus, astrovirus, and enterovirus [105].

Hepatitis A virus (HAV) is a member of the *Hepatovirus* genus in the family *Picornaviridae*. It has a positive-stranded RNA with a size about 7.5 kb. HAV can cause hepatitis, especially among infected adults, but gives mild disease in children. No chronic liver diseases caused by HAV has been reported. Almost everyone who recovers from an HAV infection obtains a lifelong immunity. However, an HAV infection could lead to fulminant hepatitis or acute liver failure, or even death, though rarely [106]. Hepatitis A is one of the most frequent causes of foodborne infection and occurs sporadically and epidemically all around the world. Its spread is mostly linked to contaminated food and water. It is also recorded in groups of men who have sex with men and among persons who inject drugs [107, 108]. Currently HAV has been classified into six genotypes. Genotypes I-III infect humans, while genotypes IV –VI are simian derived [106].

HEV has to some extent similar transmission routes and symptoms as HAV, and has been described in previous chapter. HEV1 and HEV2 infect only humans and are mostly associated with waterborne outbreaks in developing countries. HEV3 has been detected in humans, and other mammals, as well as in multiple water environments. The role of water for the transmission of this genotype is still unclear.

Human adenovirus (HAdV) is a non-enveloped, double-stranded DNA virus in the family *Adenoviridae*. There are currently members in seven species, A to G, infecting humans. Each species could be further classified into different genotypes [109]. Different types of HAdVs have different tissue tropisms, and cause different clinical symptoms, including respiratory illness, gastroenteritis, and conjunctivitis. The genotypes associated with gastroenteritis are types 40 and 41 from species HAdV-F, and type 52 from species HAdV-G [110]. These genotypes have been detected in various waters worldwide including wastewater, river water, oceans, and swimming pools [111].

Human norovirus (HuNoV), is a non-enveloped positive-stranded RNA virus in the *Caliciviridae* family. Norovirus can infect persons of all ages. It is one of the leading causes of gastroenteritis worldwide. It is estimated that norovirus is responsible for around 60% of all sporadic diarrhoea cases [112, 113]. Norovirus is classified into 5 genogroups, GI to GV, of which three could infect humans, GI, GII, and GIV, and each genogroup is further divided into numerous genotypes. Most norovirus infections are asymptomatic. In symptomatic cases, the incubation period usually is about 1-3 days, and the infection will recover within 2-5 days [114]. Severe outcomes of norovirus infections are common among infected elderly and in immunocompromised individuals [115, 116].

Rotavirus is a non-enveloped 11-segmented double-stranded RNA virus from the *Reoviridae* family. Rotavirus is the most common cause of severe diarrheal disease in young children worldwide. Its infection accounts for almost 40% of hospital admissions of children with diarrhoea and 200,000 deaths throughout the world [117]. Adults can also be infected, but the disease is usually subclinical or mild. Rotavirus causes more severe diseases and much more deaths than other enteric viruses. The majority of the deaths are due to dehydration and are reported from developing countries, where access to rehydration therapy is poor [118]. Currently four oral, live, attenuated rotavirus vaccines are available and prequalified by WHO. All four vaccines are considered highly effective in preventing severe gastrointestinal disease. The number of rotavirus-associated hospital admissions has significantly declined since the introduction of these vaccines [117].

Astrovirus is a positive-sense single-stranded RNA virus from the *Astroviridae* family. This family is classified into two genera, *Mamastrovirus* and *Avastrovirus*, based on their hosts. Those infecting humans belong to the genus *Mamastrovirus*. Thus far, three divergent groups of human astrovirus (HAtV) have been described, as classic HAstV, HAstV-MLB, and HAstV-VA/HMO [119]. HAtVs is considered as one of the leading agents of viral acute gastroenteritis in children worldwide. It typically induces a mild, watery diarrhoea that lasts 2 to 3 days, associated with vomiting, fever, anorexia, and abdominal pain, but could be dangerous to immunocompromised individuals and elderly [119]. There is no vaccine available against

astrovirus so far.

In addition to the above-mentioned enteric viruses, other viruses are also shown to cause human gastroenteritis, and were found in sewage, as sapovirus, enterovirus, and parechovirus. Sapovirus, like norovirus, is a member of the *Caliciviridae* family. Since it was first detected in human diarrheic stool samples in 1976 [120], it has been shown to cause both sporadic cases and outbreaks worldwide, and can infect and cause disease in humans of all ages [121]. Enterovirus (EV) is a member from the *Picornaviridae* family. There are 15 species of enterovirus, designated EV-A-L. Members in EV-A-D infect humans, and there are up to 63 different types in each species. Currently, 116 enterovirus types have been classified in EV-A-D, some of the best known are poliovirus, coxsackievirus, and echovirus. Apart from gastrointestinal infections, enteroviruses also cause multiple symptoms varying from mild respiratory disease, hand-foot-and-mouth disease to more severe diseases like pleurodynia, pancreatitis, meningitis, encephalitis, and paralysis [122, 123]. Human Parechovirus (HPeV) also belongs to the *Picornaviridae* family, and forms its own genus *Parechovirus*. HPeV replicates in the respiratory and gastrointestinal tract and primarily infects infants and young children. There are 18 genotypes of HPeV, with types 1 and 3, often isolated from severe cases [124, 125].

In recent years, more viruses have shown links to viral gastroenteritis. These viruses are regarded as potential pathogenic enteric viruses, such as aichi virus and torovirus. Aichi virus, belongs to the *Picornaviridae* family. It was initially reported from Japan in 1989 [126]. Since then, it has been detected in stools from diarrheic patients, but its prevalence is too low to certify an association with diarrhea, it has however been isolated from several cases of human gastroenteritis [127, 128]. Torovirus is recently classified in the *Tobaniviridae* family within the order *Nidovirales*. An association has been identified between torovirus and gastroenteritis in children and immunocompromised hospitalized patients as well as in previously healthy patients [129]. Since very few studies have reported torovirus detection, the true pathogenesis and prevalence of this virus is still unclear.

1.2.1.3 Other known and unknown viruses in wastewater

The application of next generation sequencing (NGS) technique brings a revolutionary change in identification and discovery of viruses. An increasing number of novel virus species are discovered each year. Recently, NGS was also applied for identification and discovery of viruses in sewage, which provides a better understanding of the viral diversity [130-132].

Viruses found in raw wastewater can infect humans, animals, plants, algae, and bacteria. Among them, bacteriophages are dominant, which is similar to other microbiomes that have been studied. Bacteriophages from the *Microviridae*, *Myoviridae*, *Podoviridae*, *Siphoviridae*, and *Inoviridae* family account for the largest proportion of bacteriophages, and most of them infect enterobacteria or lactococci [132]. Some bacteriophages, such as somatic and F-specific coliphages, are proposed as viral indictors for monitoring the water quality, since they have similar composition, morphology, survival rate, and size as many human enteric viruses. They

are thus considered to be better predictors than traditional fecal indicator bacteria (FIB) [133].

Plant RNA viruses are dominant in human feces. They may thus enter into aquatic environment, like sewage. Previous studies have shown that most of the known eukaryotic viruses in raw sewage were plant viruses [132, 134]. In raw sewage, plant viruses from many virus families were identified, as from the *Virgaviridae*, *Tombusviridae*, *Alphaflexiviridae*, *Betaflexiviridae*, *Partitiviridae*, and *Tymoviridae* families. One example is pepper mild mottle virus (PMMoV), which is prevalent in human feces and frequently detected in aquatic environments in relative high concentrations. This virus recently has been proposed as a surrogate of human enteric viruses for water quality assessment or detection of fecal pollution [134-136].

Apart from the above described human enteric viruses, still many virus species detected in raw sewage are associated with human diseases, as human papillomavirus, polyomavirus, human picobirnavirus, and salivirus. Human papillomavirus and polyomavirus are two groups of oncogenic viruses with tropism for skin. These viruses have been detected in urban sewage worldwide [130-132]. The observed abundance and wide dissemination of these two viruses in water environments raise the concern that these oncogenic viruses have potential of waterborne transmission. Further efforts focusing on the occurrence and quantity of these viruses in different water environments, and potential risk of leading to human diseases are essential [137].

1.2.2   Virome in drinking water

Drinking water is more directly related to human health compared to wastewater. One target of the new 2030 Sustainable Development Goal is "clean water and sanitation". More stringent regulations are implemented for monitoring of water quality in drinking water sources and in drinking water treatment plants (DWTPs) in order to supply safe, reliable drinking water to the communities. The presence of human enteric viruses in drinking water could pose a risk to local populations and is regarded as a global public health problem. The understanding of the virome in different water and the efficiency of the treatment to remove viruses during each purification process in DWTPs are needed.

1.2.2.1 Drinking water treatment plant

There are some similarities between the treatments used in WWTPs and in DWTPs. The incoming raw water into the DWTPs is treated with mechanical and chemical processes, but usually without biological processes, to produce safe drinking water. The treatments differ in different communities depending on the water sources. Groundwater normally is much cleaner than surface water and therefore needs less treatment. Surface water is not so clean and needs more advanced purification processes. Even for the same kind of water, the treatments may differ depending to the quality of raw water.

In most DWTPs, the raw water is taken from groundwater or surface water, like lakes, rivers, and streams. The raw water is pretreated by screening to remove large objects, followed by

addition of lime and chlorine in some DWTPs. This will soften the water and change the pH to prepare it for further treatment, and lower the growth of pathogens [138]. The pretreated raw water is then treated with chemical coagulation, usually aluminum sulfate and other chemicals are added to attract dirt and other particles in the water to form "floc" and sink to the bottom. Sedimentation is the following step after coagulation. This step will clear the water by sedimentation of heavy particles to the bottom. Thereafter, the treated water passes through carbon filters, where smaller particles, and most of organic compounds and chlorine, as well as unwanted tastes and odors are removed. For further reduction of microorganisms, the water is disinfected with ultraviolet light (UV light), ultrafilter (UF) or other methods. Before the water is pumped out into supply network, the pH is adjusted to a level that protects the pipes and a low chlorine dose is added to protect against bacterial growth.

The water is transported from the DWTPs through pipes to the communities. Usually there is a long transportation or storage of the water. Taking Gothenburg as an example, the produced drinking water is pumped through a 176-kilometer-long water pipeline network. It takes about 7 hours for the water to pass through the whole channels. In addition, there are 13 water towers around the city's heights functioning as equalizers. The water stored there is used during rush hours and for emergency events, such as a power failure. The monitoring of water quality in the water distribution networks is also important to provide clean and safe drinking water.

1.2.2.2 Viruses in DWTPs and in tap water

Drinking water plays an important role in modern societies. WHO recommends a multi-barrier approach to prevent the distribution of pathogen-contaminated drinking water and reduce the contaminations to levels not hazardous for health [139]. The multi-barrier approach is an integrated system of procedures, processes and tools to collectively minimize the risks and threats to public health from source water to tap water [140]. There are three major elements in this approach, including protection of source water, treatment of drinking water, and the distribution system. The efficiency of removal and inactivation of pollutants by each barrier is determined at the DWTPs. The required and determined efficiency of the barriers are compared to decide if additional treatment is needed. It is noted that each barrier itself may not be adequate in removing or preventing contamination of drinking water, but together they reduce the risk [141].

For reducing of microbiological contamination, there are several barriers used in DWTPs. The most common include chemical precipitation with subsequent filtration, slow filtration, primary disinfection, and membrane filtration. Traditionally, the monitoring and evaluation of microorganisms is based on fecal indicator bacteria (FIB), such as total coliforms, *Escherichia coli* and *Enterococci* [142]. However, some studies have shown that bacterial indicators poorly correlate to the presence of human enteric viruses. The use of FIB as the indicators for removal and inactivation also of human viruses and protozoa cysts has been questioned [143, 144]. Despite the absence of detectable bacterial indicators after treatments, there may still be contaminants, like viruses, entering into the drinking water.

The number of virus species and amount of viruses entering into DWTPs are mainly dependent on the quality of water sources. Like in WWTPs, many human viruses may enter into the DWTPs with the raw water, as norovirus, HAV, HEV, and enterovirus, which have been described in previous chapters. Many of these enteric viruses are stable and can survive for long periods in water environments. The survival rate is affected by various conditions, such as temperature and pH. It takes up to 304 days in water for 99% inactivation for adenovirus type 41 at 4°C [104, 145]. Different viruses have shown varied sensitivities to the treatments used in DWTPs. Some viruses, like adenovirus, are resistant to UV light [146, 147], while MS2, a common used viral indicator, is efficiently removed by filtration [148]. There is no single universal treatment method with high capacity, that can be applied for removing all viruses. Thus multiple barriers or treatments are needed.

According to a WHO report, there were still 2.2 billion people around the world who did not have access to safely managed drinking water in 2017. It is estimated that about 485,000 people die each year from diseases transmitted by contaminated drinking water, mostly in developing countries [149]. In developed countries, conventional water treatment techniques and additional disinfection are applied for drinking water. The amount of most human pathogenic viruses is lowered to undetectable level by traditional methods. Outbreaks or deaths caused by contaminated drinking water is therefore rare. However, in some situations, as during malfunction of the disinfection of viruses in DWTPs or when wastewater may enter into the drinking water supply network, viruses could pass through and end up in tap water. Waterborne viral outbreaks due to contaminated drinking water have been reported from several countries [150-153].

In Sweden, dozens of waterborne viral outbreaks have been documented during the last decades. Almost all of them were associated with norovirus, and a few with rotavirus [154]. Diverse norovirus genotypes were identified from patient samples during the outbreaks, with GI strains being predominant [154-156], which suggested that norovirus GI strains are more stable in water than other genotypes. The epidemiological analysis during these outbreaks showed that the consumption of municipal drinking water was a high risk factor. However, the attempts to detect norovirus strains from the drinking water failed, suggesting the shortcoming of conventional molecular detection methods for identifying viruses in low amount [156]. Besides norovirus, there are few records and little understanding of outbreaks caused by other enteric viruses. Identifying and addressing risks in the drinking water systems is important and urgent in order to prevent the potential virus outbreaks in the communities.

1.3 Virome in swine reservoir

As described before, HEV3 and HEV4 infect both humans and a number of other mammals, as domestic pigs, wild boars, deer, goat, cow, and rabbits. Among these species, domestic pigs and wild boars are the main reservoirs for HEV3. Pork meat is an important food resource. It is estimated that about 24 kg of pork meat is consumed per person each year in Sweden [157]. In addition, there is an increasing wild boar population in Sweden, which leads to more and closer contacts between humans and wild boars. The understanding of the prevalence and

characteristics of HEV in swine populations is crucial to prevent zoonotic HEV transmission from domestic pigs and wild boars to humans. The transmission of other viruses, besides HEV, from swine to humans, and between wild boars and domestic pigs are not clear, but the possibility cannot be ignored. Thus, the knowledge of the virome in swine is important to understand and possibly prevent transmissions.

1.3.1   HEV and other viruses in domestic pigs

Human HEV infection through cross-species transmission has been proved [158], and domestic pigs are the main reservoirs in developed countries. Studies have investigated the genetic relationships between HEV strains from humans and domestic pigs and found some genetically close strains. Understanding the circulation of different HEV strains in the swine populations is needed to comprehend the routes of transmission.

Investigations of the HEV prevalence both at the pig farm level and at the individual level have been conducted in many parts of world. In Sweden, the HEV RNA prevalence was 72.7% in 22 randomly selected pig farms, and in 29.6% of fecal samples from piglets in the farms [159]. In the other three Scandinavian countries, the HEV RNA prevalence ranged from 38% to 83% at the pig farm level, which is close to that in Sweden [160-163]. The prevalence of anti-HEV antibodies in pigs is also high in the Scandinavian region. More than 90% of the pig farms had pigs with anti-HEV antibodies, and 73% to 87% of those pigs had anti-HEV antibodies [160-163]. Although different HEV prevalence in domestic pig populations have been reported from different areas, it is difficult to compare these data since the sampling strategy, detection method, type of farms, type of samples, and the age of tested pigs varied between each study. Still, frequent detection of HEV suggests that HEV is constantly circulating in the pig farms. This knowledge is crucial for assessing HEV infections in domestic pigs.

Why did some pig farms have a higher HEV prevalence than others? Risk factors associated with this difference were summarized recently [164]. The type of pig farm was identified as a risk factor. Currently, several types of pig farms exist in the pig farming industry, as organic farms, conventional closed farms (keeping the sow), and conventional non-closed farms (purchasing gilts). It is shown that HEV prevalence is significantly higher in organic farms than other types [165]. This may be explained by the high frequency of direct or indirect contact with other HEV reservoirs, like wild boars or rats. Some other farming practices, such as putting piglets from several broods together at the nursery stage, inadequate cleaning between each batch of pigs, or poor hygiene conditions during the rearing [166], are also regarded as risk factors for high HEV prevalence. It is known that HEV infected pigs shed virus particles to environment by feces or urine. The virus can accumulate and be persistent in the environment of the pigs. Healthy pigs could be infected by frequent contact with the contaminated environment. The pig farm scale (the number of pigs and sows) was also shown to influence HEV prevalence. However, different trends were reported. One study from China showed that the HEV seroprevalence ranged from 78 to 100% in the large pig farms (610–1,500 pigs), which was higher than that in small scale farms (52-120 pigs) where only 0–29% of pigs were anti-HEV positive [167]. Another study from a nearby province in China showed that the HEV

seroprevalence in larger pig farms (approximately 1,000 sows) was slightly lower than in smaller farms (approximately 20 sows) [168]. The number of pigs may not be the true reason for this difference, but the pig feeding density and the utilized farming practices could contribute to it. Besides these risk factors, biosecurity measures, as requiring shower-in and providing boots for visitors, were associated with significantly reduced risk for HEV introduction [169]. The identification of risk factors associated with high HEV prevalence in pig farms would help to better control the HEV infection in pigs.

In most countries, newborn piglets stay with their mother for 4–6 weeks. Thereafter they are weaned and transferred to another section of the farm together with pigs of the same age, where they stay until they are 3 months old. Afterwards, the piglets are transferred to fattening stables until they are slaughtered. One study showed that the average age of the piglets when they got HEV infected was about 2 months, with more than 80% of infected between the ages of 30 and 90 days [170]. This is due to the newborn piglets being protected by maternal antibodies, which are disappearing after 1–2 months [171, 172]. The piglets are then at risk of getting infected with HEV or other viruses. After being HEV infected, there is normally a 1-week latency period before the piglets start shedding viruses [173, 174]. The shedding period can last for several days or up to 1 month based on the infection dose and immune condition. Morgane et al. performed a meta-regression analysis using data from 31 studies and showed that the probability of fecal shedding peaked around 3-month old piglets and the prevalence of shedding pigs at slaughter age (commonly at 6 months or at the weight of 120 kg) was about 6.1% [164]. Thus, 3-month old and older pigs are recognized as the major shedding sources in pig farms and used for the evaluating the HEV prevalence and circulation in most studies.

With the production of humoral immune responses against HEV, the viremia does not last for a long period, but viral shedding in feces may continue. At slaughter age, a number of pigs are still HEV viraemic. One study from Scotland reported that up to 44.4% of tested pigs were viraemic at slaughter age [175]. The meat from slaughtered pigs are sold in markets or made into different kinds of pork products, as sausages, liver paste, dried meat floss, and pork jerky, and enter into the food chain. The presence of HEV in pork and its by-products were reported from many European countries, as in Germany, France, and Italy [49, 50, 176]. This has raised concerns of HEV transmission through consumption of contaminated food. Genetic analysis found that strains in pork products had high sequence homology to isolates from patients with acute HEV infection in same geographic region [177, 178], which support the assumption that contaminated food is a possible source of zoonotic HEV infection.

Since contaminated pork products pose a threat for HEV transmission, there is a need to surveil and control HEV in domestic pigs and also in the food chain. Several control measures are proposed to fulfil this task [164]. The first measure is to control the risk factors that could increase HEV prevalence. These risk factors have been described in previous paragraph. Among them, a good farming practice and hygiene is relatively easy to accomplish and can effectively reduce the risk for HEV. Secondly, a good structure for the pig production network could be helpful to prevent the spread of HEV. Some pig farms purchase gilts from other farms, with a risk of introducing new viruses. Surveillance for HEV at pig farms, slaughterhouse, and in food chain is needed. This surveillance will provide continuous data on HEV prevalence and

its possible fluctuation. This may help the authorities or stakeholders to take actions for preventing HEV and other viruses to enter into the food chain. It could also help to identify dynamics and factors influencing on variations of HEV infections. However, since most of the above mentioned risk management measures are not implemented in most countries, the risk of zoonotic HEV infection through contaminated food cannot be overlooked.
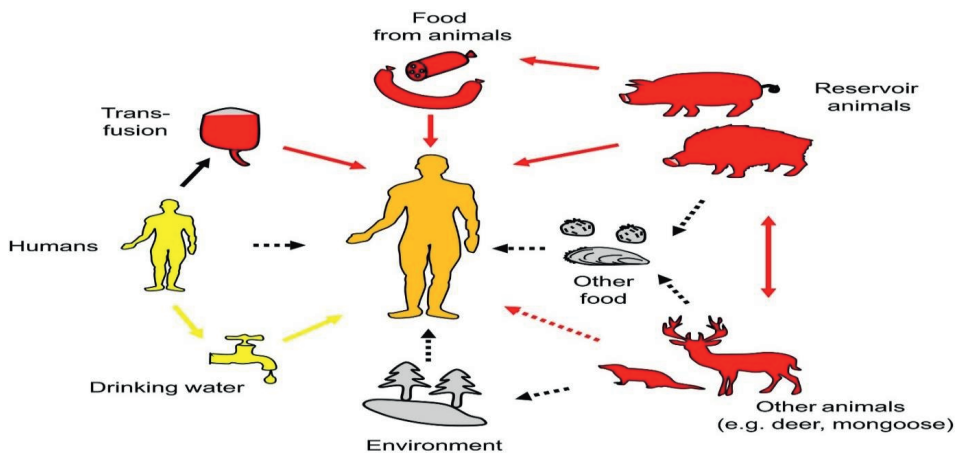
Figure 3. Transmission routes of HEV between humans, domestic pigs and wild boar. Bold arrows are routes proved, and dotted arrows are rarely shown or only suspected. Yellow lines are routes for HEV1 and HEV2, and red lines are routes for HEV3 and HEV4. Cited from C. Spahr et al. with permission [179].

Besides HEV, domestic pigs can also be infected by many other viruses. The most investigated are classical swine fever virus (CSFV), african swine fever virus (ASFV), porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus, and porcine enterovirus. These viruses can cause more severe damages to the pig farming industry than HEV. African swine fever caused by ASFV is one of the most important infectious diseases threatening pig production. This virus was first described from Kenya, then started to expand to the rest of world. Recent ASF outbreak in Asia led to huge economic losses in affected countries [180]. It has also been found in the European Union, as in Poland, Latvia, and Lithuania [181]. Considering its high virulence to pigs, monitoring of ASFV should be a priority for Europe. Another example is swine influenza virus. It has a worldwide distribution and causes acute upper respiratory diseases in pigs. The most commonly subtypes are H1N1, H1N2, and H3N2. Normally, swine influenza virus causes regular outbreaks in pigs, and does not infect humans. However, sporadic human infections caused by "variant" influenza viruses occurred. This new variant virus can be transmitted easily from person-to-person resulting in an influenza pandemic [182]. The recent pandemic in 2009 was caused by (H1N1)pdm09. An estimated of 10-200 million people got infected, and 18,500 died due to this outbreak according to a WHO report [183]. This led to an increased concern about the transmission of swine viruses to humans. Surveillance of swine influenza virus in pig populations may thus serve as an early warning for next possible swine influenza pandemic. Another virus, PRRSV, causes porcine

reproductive and respiratory syndrome (PRRS), which leads to reproductive failure in breeding stocks and respiratory tract illness in young pigs. This is regarded as one of the most economically significant swine diseases worldwide. Studies have shown that PRRSV co-infection with HEV or PRRSV infection prior to an HEV infection could delay the anti-HEV immune response, increase the amount of virus shedding, and extend the shedding period, ultimately enhance HEV transmissibility [164]. Therefore, the understanding not only HEV, but also the whole virome in domestic pig populations is necessary.

1.3.2   HEV and other viruses in wild boars

Wild boar (*Sus scrofa*) is one of the most widely distributed large mammals in the world. Its territory ranges from Western Europe to Eastern Russia, China, Japan, and throughout the Southeast Asia, and also been introduced into Australia and North America [184]. In Europe, the number of wild boar is increasing. Since the number of hunters remain relatively stable or decline in most countries, recreational hunting is not enough to limit the wild boar population growth. Other factors, as mild winters, reforestation, and intensification of crop production, lead to a steady growth of the number of wild boar throughout Europe [185, 186]. This animal has existed in Sweden for thousands of years. They were extinct during 18th century, and then reintroduced and kept in enclosures, from where they escaped. Since then the wild boar numbers increased rapidly, and there are now more than 150,000 wild boars in Sweden [187]. As the wild boar numbers continue to grow, more human-wild boar conflicts occur, especially in agriculture and in traffic. The wild boars are entering into gardens, villages and farm lands to seek for food, and are also changing the local ecosystems due to their special living habits [187, 188]. The number of wild boar-vehicle collisions rose from about 50 case in early 2,000, to about 1,000 in 2005, and more than 4,000 cases in 2012 in Sweden [189]. In addition, the wild boar is the main reservoir of many pathogenic viruses. Direct or indirect contact with domestic pigs and humans may transmit the viruses, and cause damage to the livestock industry.

Wild boars are genetically close to domestic pigs. Taxonomically they are from the same species, but belong to different subspecies. Domestic pig and wild boar share some similarities in HEV infection, as being infected with genotypes HEV3 and HEV4. Studies from Japan also found additional genotypes HEV5 and HEV6 co-circulating with HEV3 and HEV4 in Japanese wild boars. These genotypes have not been identified in domestic pigs and humans [190]. As the wild boar population expands and starts to invade human communities, the more frequent contact with humans and domestic pigs enhances the risk of introducing novel HEV genotypes to humans. Although the transmission ability of HEV5 and HEV6 to humans is not clear, their potential zoonotic transmission should not be ignored. Other new not yet identified genotypes may also be infecting wild boars in Europe.

The prevalences of anti-HEV antibodies and HEV RNA in wild boar populations have been studied in several European countries. The seroprevalence against HEV ranges from 10% to 44% in Europe [25, 54, 191-193]. One study estimated a prevalence about 15% among Swedish wild boars [194]. Several studies have also investigated the HEV RNA in wild boars. A low HEV RNA prevalence ranging from 2.5% to 25% was reported from most European countries

[195-198], but a higher prevalence up to 68% was reported from Germany [25]. The difference of HEV prevalence among wild boars across Europe may be explained by geographical location, climate, and human life habits. Sequence analysis have shown that all infections in wild boars are caused by HEV3 strains of different subtypes. HEV3 is divided into two major clades based on phylogenetic analysis, clade I with six subtypes HEV3a, b, c, h, i, and j, and clade II with three subtypes HEV3e, f, and g [159, 199]. The identification of the different HEV subtypes in the wild boar populations would be helpful to understand the relations between strains found in wild boars, domestic pigs, and humans.

The routes of HEV transmission from wild boars to humans are similar with that from domestic pigs. It is mainly through direct contact or consumption of under-cooked wild boar meat or by-products. HEV infected wild boars can shed viruses in feces and urine, and subsequently contaminate soil, river, or berries in the forests. This route of HEV transmission to humans has been suspected (Figure 3). Several studies have shown that forestry workers and hunters have higher HEV seroprevalence than control groups [54, 191, 200]. Swine HEV3 strains have been detected on strawberries in the fields [43], but direct evidence is still needed to confirm this route of transmission.

Until now, a total of 179 different virus species were identified in wild boars according to data from the Virus-Host database [201]. All pathogenic viruses described in domestic pigs can also been found in wild boars. Two viruses that have been actively surveilled in the Swedish wild boar population are Aujeszky's disease virus and classical swine fever virus. The former causes Aujeszky's disease that severely affects pig production worldwide. It has been well controlled in the domestic pig population, but is still widespread in the European wild boar populations. Classical swine fever virus causes similar clinical symptoms as ASFV and is highly contagious. It is transmitted by direct and indirect contacts between animals. Cases have been reported from both domestic pigs and wild boars in several European countries [202]. A spread of ASFV to EU is ongoing and involves wild boars. Direct and indirect contacts between domestic pigs and wild boars hamper the control of this disease. Both passive and active surveillance conducted recently in Swedish wild boars could not identify these viruses. Since the part of Sweden which is populated by wild boars is surrounded by sea borders, the risk for infected wild boars migrating into Sweden is low. However, wild boars could gain diseases from other routes, as access to infected meat or animal products in garbage, or through transmission from other animals or humans [203]. All these monitored viruses have been present in countries close to Sweden, thus the further monitoring and investigation in wild boar population is crucial in order to prevent the spread of these viruses.

2. AIMS

GENERAL AIM

The overall aim of this thesis was to investigate the prevalence of HEV and other viruses in different types of water and animals to get some understanding of their dissemination into the environment.

SPECIFIC AIMS

Paper I

To investigate the HEV prevalence and genetic relationship between HEV strains in Swedish and Spanish wild boars.

Paper II

To investigate the HEV prevalence in Swedish pigs and the genetic variability of HEV strains between different pig farms, and to determine if the circulating strains change over time.

Paper III

To investigate the virome in incoming and effluent sewage and the efficiency to eliminate or reduce the amount of viruses in a sewage treatment plant processed by conventional treatment with and without additional ozone treatment.

Paper IV

To evaluate the efficiency of removing viruses during the purification process from raw to drinking water, and to investigate the virome in the different water.

Paper V

To characterize the seasonal pattern of common enteric viruses in wastewater and the virome in treated effluent water during one-year follow-up at a WWTP.

3. MATERIALS AND METHODS

3.1 Study design and samples collection

In this thesis, water samples, animal samples, and human patient samples were collected for analysis. Water samples included environment water (from rivers and lakes), water from wastewater treatment plants and drinking treatment plants, and tap water. Animal samples were Swedish and Spanish wild boars, and Swedish domestic pigs. Patients infected with HEV of unknown source of infection, and patients with typical diarrhea symptoms seeking medical help were included for the analysis. The detailed sample information for each study is described below.

Paper I

In this study, serum and/or fecal samples from Swedish and Spanish wild boars were collected for comparison. Here we collected both serum, fecal and liver samples from 134 Swedish wild boars, fifty-six were hunted in 2015 and the remaining 78 were hunted during the second half of 2015 and during 2016, by cooperating with local hunters from ten different regions with up to 600 km distance from each other. In Spain, 74 wild boars with only fecal sample, 57 with only serum sample, and 133 with both serum and fecal samples were collected within the Barcelona Metropolitan Area between April 2015 and September 2016. After hunting or capture, the age of the wild boar was estimated, and then the samples were stored in RNAlater RNA Stabilization Reagent and sent to the Clinical Microbiology Laboratory (CML) for the analysis.

Sera from 48 Swedish human cases with unknown source of their hepatitis E infection were collected and subsequently analyzed for routine anti-HEV antibodies and HEV RNA tests. All the samples collection was approved by either the ethical committee on animal research in Uppsala, Sweden or the Regional Ethical Review Board in Gothenburg.

Paper II

Fecal samples from three-month old piglets were collected in the growing stables from 30 Swedish pig farms, distributed in central and southern Sweden. The first sampling was carried out between April and early June 2013, a total of 180 fecal samples were collected. The second sampling was conducted in the same pig farms but after 12-17 months, a total of 183 fecal samples were obtained between late April and September 2014. In this study, four types of pig farms, including organic farms (nine farms), conventional closed farms with their own recruitment of gilts (seven farms), conventional satellites in a sow pool sharing sows (seven farms), and conventional non-closed farms purchasing gilts (seven farms), were investigated. Meanwhile, HEV strains in serum samples from 36 Swedish human cases with endemic hepatitis E of unknown source of infection in 2011-2017 were sequenced for further comparison.

Paper III

All the samples were collected from Knivsta WWTP, located 50 km north of Stockholm, Sweden. This WWTP received primarily household wastewater from up to 12,000 person equivalents with a typical water flow of 150 m^3/h. The received sewage was treated with traditional activated sludge treatment and additional ozone dose of ca 6 mg/L. The treated water was then discharged into recipient river.

In total, three groups of water samples were collected between week 49 and week 52 (Group I from November 30 until December 4; Group II from December 8 until December 12; and Group III from December 19 until December 22) in 2015 were analyzed. In each group, flow-proportional 24 h composite samples of influent (5 L per sample; day 0), effluent after conventional treatments (10 L), effluent after ozonation (10 L) and outlet sewage (10 L; day 4) were collected and adjusted for the flow rate to represent the "same" water.

Paper IV

In Gothenburg, the raw water is transferred and processed at two DWTPs, Alelyckan and Lackarebäck, to produce drinking water. The raw water is first treated by conventional chemical flocculation, sedimentation, and activated carbon filtration methods. Additional disinfection is achieved using a dose of 40 mJ/cm² of ultraviolet light (UV light) at Alelyckan, and using ultrafiltration (UF) membrane with a nominal size of 20 nm at Lackarebäck. Before the water is pumped out to the supply network, pH is adjusted to a level that protects the pipe, and chlorine and chlorine dioxide are added to inhibit bacterial growth and to control lake related odor.

In Lackarebäck DWTP, Nano-Ceram cartridge filter was connected to the flow of water, 5,050 L of water at a stable flow rate of 2.9 L/min for 29 hours after activated carbon filtering and 10,350 L of water at a stable flow rate of 1.45 L/min for 119 hours that after having passed UF membranes were filtered. In Alelyckan DWTP, the same sampling device was applied, with 6,000 and 7,100 L of water before and after UV treatment were filtered for 48 hours with flow rates of 2.08 L/min and 2.48 L/min, respectively. Twenty litres of raw water to be treated at Lackarebäck DWTP was filtered through Nano-Ceram cartridge filter at an average flow rate of 2.5 L/min. In addition, between 1,168 and 1,438 L tap water from CML lab, which is supplied by both DWTPs, was filtered by connecting Nano-Ceram cartridge filter to the tap during three consecutive nights.

Paper V

All the influent wastewater and effluent wastewater samples were collected from Ryaverket WWTP, which is located in Gothenburg, Sweden. A total of 26 influent wastewater samples were collected every second week between 26th December 2016 and 18th December 2017. The influent wastewater was sampled over 24h using a fixed-site sampler, and the volume was proportioned to the inflow of influent wastewater. The daily sample was stored at 4°C and

pooled into a weekly sample, then stored at -20°C. Meanwhile, a total of 11 effluent water samples were collected monthly starting from the end of March, 2017 until the end of January, 2018. The Nano-Ceram filter was connected to the flow after treatments at stable rate of 8 L/min and the sampling last for about 24 hours.

In this study, the samples from 2,846 patients in the Västra Götaland area showing diarrhea symptoms and seeking for medical help during 2017 were included in this analysis. Their fecal samples were send to CML and analyzed with routine real-time PCR detection of enteric viruses, including norovirus GI, norovirus GII, norovirus GIV, adenovirus, rotavirus, sapovirus, and astrovirus.

3.2 Virus concentration and nucleic acids extraction

In **Paper I and II**, fecal samples from wild boars and domestic pigs were pretreated before further test. Basically, 1 gram of stool sample was weighted and homogenized in tubes containing glass beads in phosphate-buffered saline (pH 7.4) and thereafter centrifuged. The supernatant was collected for nucleic acids extraction. The serum samples in **Paper I** were directly processed for nucleic acids extraction.

In **Paper I**, twenty-three liver samples from HEV RNA positive Swedish wild boars were selected for NGS analysis. About 100 mg of wild boar liver was cut into small pieces and added into Precellys Lysing Tube. Tris-HCl (10mM, pH 8.0) was added and the sample was homogenize using Minilys homogenizer. The homogenized sample was centrifuged to remove cell debris. The supernatant was filtrated through a 0.45 um filter to remove bacteria.

For concentration of viruses from multiple types of water samples, an in-house virus concentration method by combining Nano-Ceram cartridge filter and ultracentrifugation was developed (Figure 4). For water samples with a high turbidity, like influent wastewater, a pre-centrifugation was necessary to remove big debris and sediments. The other types of water samples could be directly filtered twice through Nano-Ceram cartridge filters. Viruses were eluted from filter membrane with 0.2 M phosphate buffer containing 0.05 M glycine (pH 9.5). The pH of the eluent was adjusted to 7.4-7.5 to ensure that no virus inactivation occurred. The eluent was then filtered through a 0.65/0.45 μm Sartobran Capsule filter to remove remaining debris and most bacteria. After second filtration, the filtrate was ultracentrifuged. The pellet was dissolved in Tris-HCl (pH 8.0). This workflow can be directly applied for small volume of water, such as influent wastewater and environmental waters, which normally have a size of 2 L to 20 L. For large volume of water samples, as the treated water from DWTPs and WWTPs, and tap water, a Nano-Ceram cartridge filters should be connected to the water flow and processed as above described. This virus concentration method has been used in **Paper III, IV, and V**.

The virus concentration efficacy of our in-house developed method was evaluated by both quantitative qPCR and isolation on cell culture. We spiked a known amount of mastadenovirus 2 (HAdV-2) to 3.5 L raw sewage and then concentrated using above described workflow. One mL of water from virus-spiked water and each concentration step was collected and analyzed

for the amount of HAdV-2.
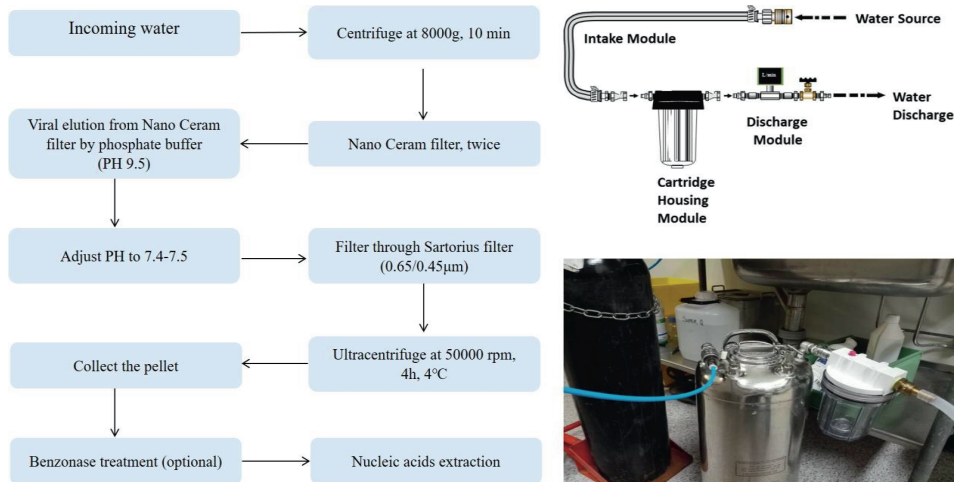
Figure 4. Brief schematic diagram of virus concentration by using Nano-Ceram cartridge filter and ultracentrifugation method.

In Paper V, the influent wastewater was concentrated by using our previously modified skimmed-milk flocculation method [204]. The reason to use this method is trying to be consistence with a previous pilot study which was conducted in the same area using the same concentration method for the incoming wastewater. This method has similar virus concentration efficiency as the newly developed Nano-Ceram filter method, but takes longer time. Briefly, a pre-centrifuge was applied to remove big debris and 10 mL acidic skimmed-milk solution containing 0.6 M sea-salt (pH 3.5) was added to every 1,000 mL wastewater sample and adjusted pH to 3.5. The mixture was stirred at room temperature for 8 hours, let it still overnight to form viruses-milk sediments. The solution was removed without disturbing the sediments. The remaining solution and sediments were centrifuged. The pellet was dissolved in phosphate buffer (pH 7.5) and 0.25M glycine buffer (pH 9,5), and stored on ice. The mixture was then centrifuged again and the supernatant was ultracentrifuged at 50,000 rpm for 2 h. The pellet was dissolved by adding 0.2 M phosphate buffer (pH 7.5) and stored at -80°C until further analysis.

The total nucleic acids in **Paper I and II** were extracted by using the NucliSens® easyMag® instrument and reagents according to the manufacturer's instructions. Two hundred fifty microliters of stool suspension and serum samples were used as input materials and then eluted with 110 µL elution buffer. The total nucleic acids in **Paper III, IV, and V** were extracted by using the QIAGEN DNA Blood and Tissue kit according to the manufacturer's instructions. Two hundred microliters concentrated water samples were used as input materials and then eluted with 200 µL elution buffer.

3.3 Virus detection

3.3.1 Serological analysis

In **Paper I**, HEV seroprevalence in 324 Swedish and Spanish wild boar serum samples were investigated by using ELISA assay. All serum samples were analysed for total anti-HEV antibodies (anti-HEV IgG and IgM simultaneously) using an HEV Ab EIA kit according to the manufacturer's instructions. The optical density (OD) value for each sample was read in an Infinite F50 microplate reader at 450 nm using 650 nm as the reference wavelength, and the cut-off value was calculated according to the manufacturer's instructions.

3.3.2 Real-time PCR detection

In Paper I and II, a one-step TaqMan RT-qPCR assay targeting the ORF2/3 region of HEV1-HEV4 was used for the detection of HEV in animal samples. For the detection of DNA viruses, such as adenovirus and parvovirus, the extracted nucleic acids can be used directly for the real-time PCR, otherwise a cDNA transcription was needed. The RNA was reverse transcribed into cDNA using random hexamer primers. A total of 21 different viruses were subsequently tested by real-time PCR from **Paper III, IV and V,** the monitored viruses were adenovirus, HAV, HEV, aichi virus, astrovirus, torovirus, sapovirus, norovirus GI, GII, and GIV, enterovirus, HPeV, rotavirus, gokushovirus, astrovirus 4, parvovirus, parvo-like virus, picobirnavirus, HAdV-41, and human pecovirus.

In Paper I, II, III, and V, a plasmid containing all viral target regions were synthesized. The plasmid was ten-fold serial diluted and used as positive control in all qPCR analyses. Then the amount of virus genomes was estimated by using the formula: (Ct value of the sample) = -3.3 * $\log_{10}$ genomes/mL+45. In **Paper IV**, The WHO International Standard (IS) for HEV with a 250,000 International Units (IU) of HEV RNA/mL was serially diluted and used as a standard for quantification.

3.3.3 PCR amplification and sequencing

In this thesis, adenovirus (**Paper III**), gokushovirus (**Paper IV and V**), and HEV (**Paper I, II, IV and V**) were selected for PCR amplification and subsequently sequencing. Nested or semi-nested PCR were performed.

The PCR products were visualized by 1.5% agarose gel electrophoresis and then purified by QIAquick PCR purification kit according to manufacturer's protocol. The purified amplicons were either in-house sequenced with the 3130xl Genetic Analyzer (**Paper I and II**) or sent to Eurofins Genomics for Sanger sequencing (**Paper III, IV, and V**)

3.3.4 Next generation sequencing

Next generation sequencing (NGS) was used to explore the virome in water and animal samples, but these samples were tested in different platforms. The influent wastewater samples from **Paper III** were sequenced on the Ion Torrent PGM platform, the others were sequenced on the Illumina HiSeq 4000 platform.

All the samples were first pretreated with Benzonase nuclease to degrade all forms of free DNA and RNA regardless of sequencing platform used.

In Paper III, DNA was extracted by using QIAamp DNA Mini Kit, and RNA was extracted by using the RNeasy Plus Mini Kit. The RNA was reverse transcribed into cDNA. Afterwards, extracted DNA and cDNA were amplified by nested PCR in triplicates. The PCR products from each sample were pooled and were sheared into 200-500 bp fragments by sonication using a Bioruptor sonication device, the size of sonication products was visualized by 1.5% agarose gel electrophoresis. The libraries were built using Ion Plus Fragment Library Kit on AB Library Builder™ System according to the manufacturer's protocol. The size of libraries was further selected to around 370 bp by using a Pippin Prep kit, afterwards the size and concentration were determined using Agilent High Sensitivity D1000 ScreenTape System on TapeStation 2200 and Ion Quantitation Kit. The libraries were diluted and pooled to reach a final concentration of 50 pM and sequenced on the Ion Torrent PGM platform.

For the other samples in **Paper I, IV** and **V**, a different NGS libraries preparation workflow was applied. The nucleic acids were extracted using DNeasy Blood & Tissue Kits after Benzonase nuclease treatment. The RNA was reverse transcribed into cDNA using Omniscript Reverse Transcription Kit. The cDNA was then amplified by nested PCR in triplicate together with the DNA sample. The first round of amplification was a touch-down gradient PCR, followed by a nested PCR amplification. The products from both DNA and cDNA amplification were pooled and purified with QIAquick PCR purification kit. The libraries were built by using Nextera DNA Flex Library Prep Kit according to the manufacturers' protocol. The quality of the libraries was checked using Qubit 4 Fluorometer and Agilent High Sensitivity D1000 ScreenTape System on TapeStation 2200. The library pool was sent to Eurofins Genomics for Illumina sequencing on a HiSeq 4000 platform.

3.4 Data analysis

3.4.1 Sequences analysis

All sequences were imported into SeqMan in the DNAStar program package version 10.1.2 for further analysis. The obtained HEV, gokushovirus, and adenovirus sequences were aligned with corresponding region of reference sequences from GenBank. The evolutionary distances were estimated using DNADIST program in the PHYLIP package, version 3.65 [205]. Phylogenetic trees were constructed using the unweighted pair-group method using arithmetic averages

(UPGMA) and the neighbor-joining (NJ) method in the NEIGHBOR program of the PHYLIP package. The trees were visualized using the program TreeView. The HEV sequences from this thesis were deposited in GenBank (Accession numbers MK333339-MK333350; MK582523-MK582474).

3.4.2 NGS data analysis

In **Paper III**, we used the Ion Torrent platform for the NGS, the data after sequencing was automatically trimmed by Ion Torrent Suite software. Then the BAM files were imported into CLC Genomic Workbench 9.5.1 for analysis. Firstly, low quality reads, reads below 30 bp and the primer sequences were removed, and then sequences were mapped to human hg19 reference genome to remove potential host genome. The unmapped reads were *de novo* assembled into contigs using CLC de novo assembler. The assembled contigs and unassembled singletons longer than 50 bp were blasted against NCBI GenBank non-redundant nucleotide database (nr/nt) using BLASTn. Those contigs and singletons that satisfied an E-value $<10^{-3}$ and HSP length >80 bp were regarded as possible virus reads and selected for further analysis (Figure 5).

The NGS in other papers were conducted on the Illumina platform, and we developed a new analysis workflow (Figure 5) since Illumina produced larger amount of data. In the new workflow, the raw data from Illumina sequencing was also imported to CLC Genomic Workbench 11.0.1 for analysis. Low quality reads were discarded and primer sequences trimmed. Then all reads were *de novo* assembled into contigs. Those contigs and unassembled singleton reads longer than 100 bp were blasted against a local genomic viral database using BLASTn. All reads satisfied a cut-off for E value $<10^{-5}$ and HSP lengths >100 bp were considered as possible virus hits and used for the second blast, which against all genomes in the NCBI GenBank non-redundant nucleotide database (nt/nr) using BLASTn. The reads that satisfied the same criteria as in the first blast were considered as significant virus hits, and further classified into virus family level.

After NGS analysis, several identified viruses (human feces pecovirus, picobirnavirus, parvovirus, parvovirus-like virus, adenovirus, astrovirus 4 and gokushovirus from **Paper III**; HEV and gokushovirus from **Paper IV and V**) were selected to validate the NGS results. All the reads were mapped to their reference sequences and then the consensus sequences were extracted. The primers and probes were designed based on consensus sequences, and real-time PCR or nested PCR were developed to confirm NGS results.

3.4.3 Statistical analysis

The significance of the differences of prevalence of anti-HEV antibodies and HEV RNA in wild boars and pigs were estimated between different wild boar populations (**Paper I**) or between different types of pig farms and different years (**Paper II**), chi-square and Fisher's exact test were applied using IBM SPSS Statistics software, $p < 0.05$ was considered

statistically significant.

3.4.4 Patient sample analysis

In **Paper V**, fecal samples from patients with diarrhea symptoms and seeking for medical help were analyzed for seven common enteric viruses by real-time PCR at CML. The number of diagnosed patients were then compared with the estimation number of potential infected patients, which was calculated based on the formula, $Y = \frac{X*Z}{10^{11}*10\%}$. Y is the number of potential infected patients per week, X is estimated number of virus genomes/L in influent wastewater, Z is the total wastewater inflow per week, 10% is estimated virus concentration efficiency of our method. It is assumed that an infected individual can excrete between 10^7 to 10^{13} norovirus, enterovirus, adenovirus, and HAV virus particles per day [206], and the median 10^{11} virus particles an infected individual excreted per week was used in this study.
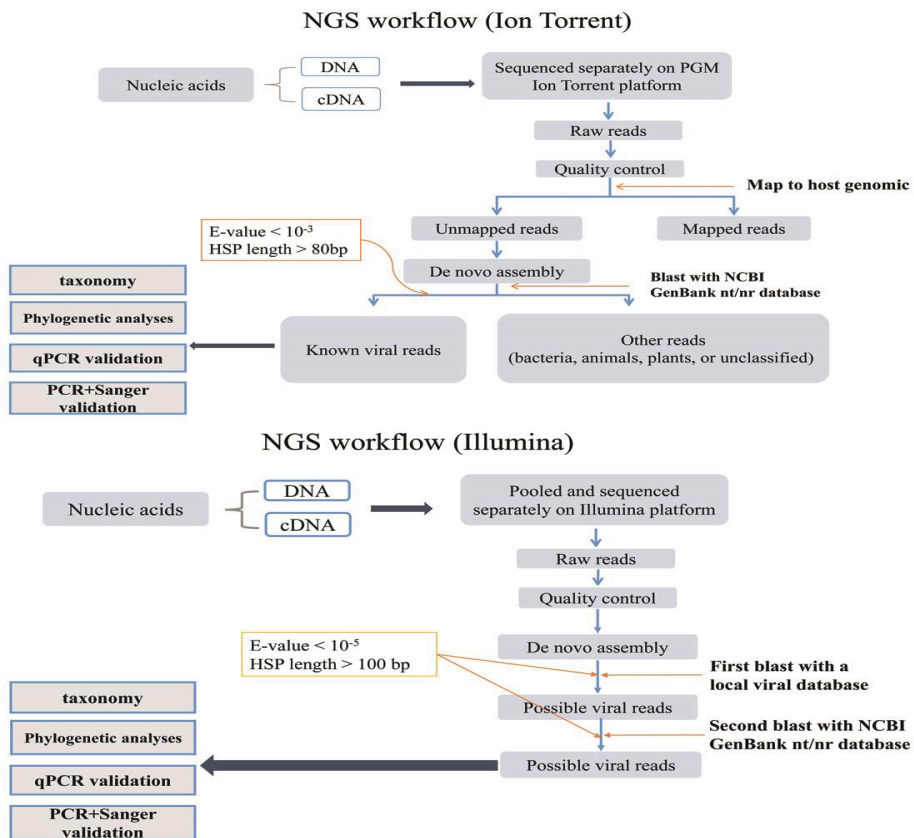
Figure 5. NGS analysis workflow for Ion Torrent and Illumina platform.

4. RESULTS AND DISCUSSION

4.1 Paper I: Genetically similar hepatitis E virus strains infect both humans and wild boars in the Barcelona area, Spain, and Sweden

Wild boar is recognized to play an important role of HEV transmission to domestic pigs and humans. In this paper, serum and/or fecal samples from 264 Spanish wild boars and both serum and fecal samples from 134 Swedish wild boars were collected. The prevalences of total anti-HEV antibodies (both anti-HEV IgG and IgM) and HEV RNA were evaluated to estimate the HEV epidemics among wild boar populations in two countries. Subsequently, HEV genotypes were determined, strains were sequenced and compared genetically with strains isolated from humans and domestic pigs to understand the risk of zoonotic transmission.

4.1.1 The prevalence of anti-HEV antibodies and HEV RNA in Swedish and Spanish wild boars

The presence of anti-HEV antibodies indicates that an HEV infection has occurred, while the presence of HEV RNA is a marker of ongoing infection. In total, 324 wild boar serum samples were analyzed for anti-HEV antibodies; 134 were from Sweden, 190 were from Spain. As shown in Table 2, the HEV seroprevalence in Spanish wild boars (112/190; 59%) was significantly higher than that in Swedish wild boars (11/134; 8%; p < 0.0001 Fisher′s exact test). This high seroprevalence in the wild boars in Collserola Natural Park and in Barcelona was consistent with that found in central (62/108; 57.4%), and southern Spain (57/99; 57.6%) [27, 207]. In other parts of Spain, the seroprevalence is lower between 5.2% and 43% [208-210]. The 8.2% seroprevalence against HEV found in Swedish wild boars collected from 2015-2016 was close to a previous investigation conducted between 2012 and 2015, with 5.2% (7/134) of the wild boars having anti-HEV antibodies [194]. This indicates a lower HEV circulation among wild boars in Sweden compared to Spain. The high seroprevalence among wild boars within the Barcelona Metropolitan Area highlights the needs for more attentions to the expanding wild boar population, and the risk for HEV transmission. Although there was a difference in seroprevalence between Spanish and Swedish wild boars, the HEV RNA prevalence in the two countries was similar (15% in Spain vs 20% in Sweden). In Europe, a wide range of HEV RNA prevalence in wild boars has been reported. Up to 68% was reported from Germany [25], whereas in most other European countries, the HEV RNA prevalence ranged from 6% to 25% [210-214]. In Slovenia, 30% of the wild boars had anti-HEV antibodies, but only one sample was HEV RNA positive (0.3%) [215]. It is worth noting that the HEV RNA prevalence among Swedish wild boars was much higher than that reported from the same areas several years ago, where only 4.2% of the wild boars were HEV infected [194]. There may have been a shift to more virulent strains, which may need further investigations.

Table 2. Number of wild boars hunted monthly in the Barcelona region between April 2015 and September 2016 and number of Swedish wild boars hunted in 2013-2015. Number of total HEV antibodies and HEV RNA reactive serum and fecal samples from the animals are given.

Origin and month and year of hunting	Number of animals hunted	Anti-HEV Number of reactive/ number of analyzed samples (%)	HEV RNA Number of reactive/ number of analyzed samples		
			Total (%)	Serum (%)	Feces (%)
Barcelona					
2015					
April	10	0/0	0/10	0/0	0/10
May	12	0/0	3/12	0/0	3/12
June	14	0/0	0/14	0/0	0/14
July	24	0/0	0/24	0/0	0/24
August	1	0/0	0/1	0/0	0/1
September	14	1/6	4/14	4/6	0/8
October	18	11/18	3/18	3/18	3/18
November	19	9/19	3/19	3/19	3/19
December	17	14/17	4/17	4/17	3/17
2016					
January	17	12/17	5/17	5/17	5/17
February	12	7/12	4/12	4/12	2/10
March	7	2/5	3/7	3/7	2/7
April	6	4/6	0/6	0/6	0/6
May	9	4/9	1/9	1/9	1/7
June	8	5/8	1/8	1/8	1/8
July	35	18/35	6/34	6/34	2/28
August	36	21/36	12/36	12/36	0/0
September	5	4/5	3/5	3/5	0/3
Total	264	112/190 (59%)	52/264 (20%)	31/190 (16%)	25/207 (12%)
Sweden	134	11/134 (8%)	20/134 (15%)	12/134 (9%)	9/134 (7%)

This study showed higher prevalence of anti-HEV antibodies but similar prevalence of HEV RNA in Spanish compared to the Swedish wild boars. Age composition of the wild boar populations and genetic differences between the HEV strains may lead to this difference. Other studies have identified age as a risk factor associated with HEV infection in wild boars. A significantly higher seropositivity was found among adults (>3 years old) and sub adults (1-3 years old) than in yearling animals (<1 year old) [207]. This may be due to a cumulative exposure to HEV, since antibodies could last for long periods of time. In this analysis, there

was a different age structure between the wild boars in the two countries. About 63% of the Swedish and 48% of the Spanish wild boars were less than one-year old. Younger wild boars are at higher risk to become infected due to low prevalence of anti-HEV antibodies. The more adult wild boars in the populations have higher anti-HEV prevalence. Although the Spanish wild boars were older than the Swedish ones, they had a slightly higher incidence of HEV RNA, which may indicate that they have a higher frequency of chronic HEV infections. This may due to genetic differences between the circulating HEV strains in the wild boar population. The different lineages of the HEV strains will be discussed in the following section.

Apart from the age and genetic difference between the HEV strains, there may be other factors contributing to different HEV prevalences in wild boars from different regions, or even in the same area. One possible factor is the sampling matrices. Several types of samples, as serum, liver, bile, lymph nodes, and feces, have been used for detection of HEV RNA in wild boars with varying results. In this study, we collected serum and feces for the analysis. HEV RNA was more often detected in serum than in feces (16% vs 12% in Spain; 9% vs 7% in Sweden), which was in line with previous studies [194, 214]. This has also been described from other animals, like moose, red deer, and domestic pigs [194, 211, 216]. For other types of matrices, it was shown that bile most often had detectable HEV RNA, followed by liver and serum/blood [25, 47]. The study from Germany showed that HEV RNA was present in up to 56% of bile samples, 38% of liver and only in 16% of serum samples [25]. Another factor could be the detection method. The common methods to detect HEV RNA are conventional one-step RT-PCR, nested RT-PCR and real-time RT-PCR, but their sensitives varied. One study showed that the two-step RT-PCR could increase the HEV RNA detection rate to 25.8% from 11.6% by one-step RT-PCR [214]. In another study, the prevalence of HEV RNA was increased from 3% to 8% by real-time RT-PCR compared with conventional one-step RT-PCR [211]. Therefore, direct comparison between the prevalence of HEV given in different studies may not be comparable, and a normalization of detection methods should be developed.

Our analysis found that 55% (26/47) of Spanish and 90% (18/20) of Swedish HEV RNA positive wild boars lacked anti-HEV antibodies. Two possible reasons may lead to this discordance. As mentioned before, a high proportion of the tested wild boars, especially in Sweden, were less than one-year old, and the HEV infection is probably in early stage. HEV RNA can be detected in the blood and feces during the incubation period, and there is a delay of several weeks before the humoral immune develops. Another possible reason is that the HEV infection did not induce or delayed the antibody response. Similar patterns have been observed in blood donors from Sweden and Germany, whose plasma were anti-HEV negative despite HEV viremia for over 90 days. Further analysis suggested that variation at the start codon for the ORF3 protein of the infected HEV strains may be the cause for immune escape [217]. Although it was shown that these strains may delay the immune response, there is no knowledge about their virulence, which needs further investigation.

4.1.2 Phylogenetic analysis of Spanish and Swedish HEV strains

A partial ORF1 region was amplified in the HEV RNA positive samples. Sequences could be

obtained from 12 of 52 Spanish and 9 of 20 Swedish wild boars. Phylogenetic analysis showed that all strains belonged to HEV3 and there were multiple HEV3 subtypes in both wild boar populations. In the phylogenetic tree, nine Spanish and two Swedish strains were found in clade 3I (Figure 6). All these Spanish strains were on the branch formed by subtype 3c/i. Five of them were genetically similar and formed a unique clade, indicating that they had the same origin. Another three Spanish HEV3c/i strains were on the same branch, but intermixed with strains isolated from Spanish and Swedish humans. The ninth Spanish HEV3c/i strain was genetically similar to strains from chronically infected patients. Whether some strains in wild boars are more likely to cause chronic infection than other subtypes or lineages is still unclear. Since many chronic carriers in Sweden are more often infected with HEV3c/i than with the more common HEV3f, there may be differences in the inducing of humoral immune response between different HEV subtypes. It is therefore important to inform, especially immune-compromised patients to avoid eating insufficiently cooked meat or meat products from pork, but also from wild boars [218].

Another three Spanish strains and seven Swedish strains were classified as subtype 3f in clade 3II. In the phylogenetic tree, the strains from both the Spanish and Swedish wild boars were intermixed with strains from Swedish domestic pigs and patients, as well as from patients from other European countries. HEV3f is not only one of the most common subtypes infecting European wild boars, but is also prevalent in pigs and humans in many European countries [25, 54, 196, 219]. The finding of HEV3f in Swedish wild boars was consistence with a previous study conducted in Sweden 6-7 years ago [159]. The rapid expansion of the wild boar population in Sweden is increasing the contacts between wild boars and local livestock, especially for pigs in ecological farms. This will lead to an increased risk of HEV transmission from wild boars to pigs, and vice versa. This has occurred in Sweden, as was shown in this study, with five Swedish wild boar strains genetically similar to strains from Swedish domestic pigs. Two Swedish wild boar strains were on the same branch with strains from Swedish patients indicating that zoonotic transmission from wild boars to humans may be a common way of HEV infection.

Among the HEV3f strains, the Spanish wild boar strains had similar pattern as the Swedish strains. One Spanish HEV3f strain formed a clade with strains from Swedish and Bulgarian patients in the phylogenetic tree. One route may be through the production of meat or liver products from infected Spanish wild boars, which were sold to other European countries. Although subtype HEV3f is more frequently detected in Spain than the other subtypes, emergent HEV subtypes, as HEV3r, have been recently found in Spanish wild boars and Spanish residents [207]. Monitoring HEV strains infecting wild boars will give information on the emergence of new subtypes and strains. It will also indicate on the most common routes of transmission between different host species.
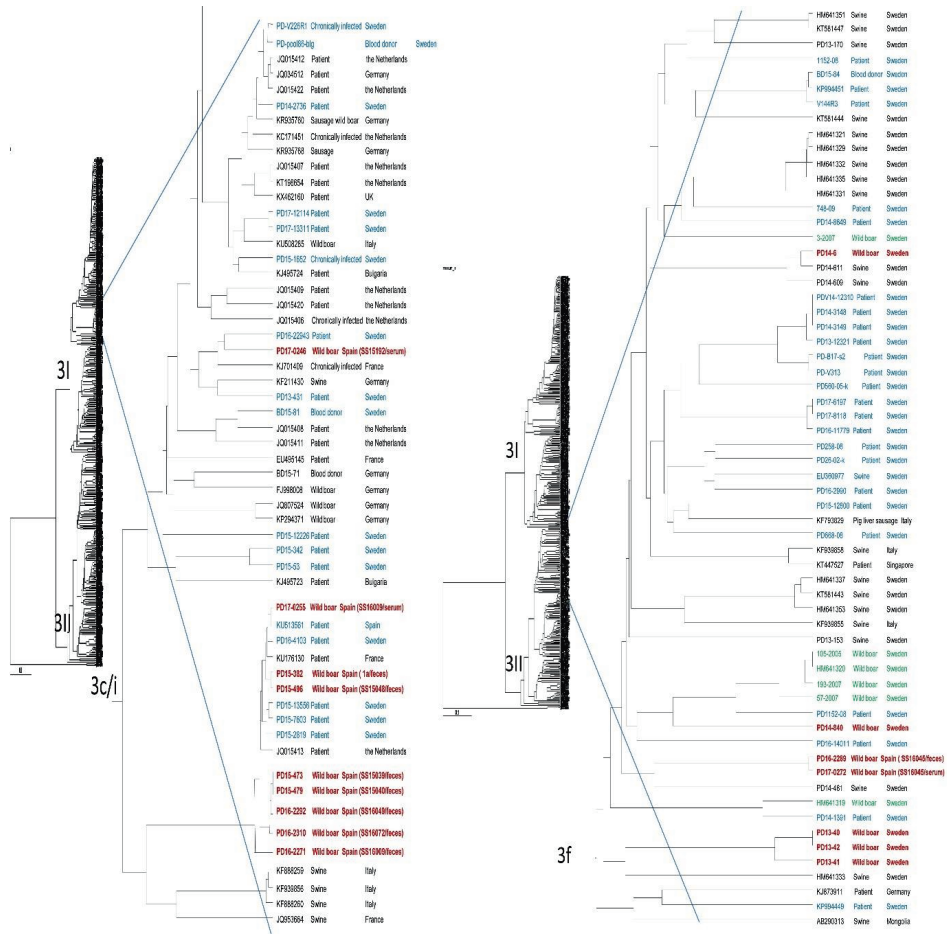
Figure 6. Phylogenetic tree based on 325 nucleotides of partial ORF1 for genotype 3. The branches with 3I subtype 3c/i and 3II subtype 3f strains are enlarged. Accession numbers and origin are given at the nodes. The strains from wild boars sequenced in this study are marked in red and from previous investigation are marked green. Strains from humans infected in Sweden are marked in blue.

4.1.3 The virome in Swedish wild boars

Liver samples from 23 Swedish wild boars were used to investigate the virome in the wild boars. The nuclei acids were extracted and used for PCR amplification and NGS as for the water samples in Papers IV and V. Most of the reads obtained were host derived, but between 0.02 to 0.6% of the reads were virus sequences. The data is still to be compiled. At least 27 different viruses were identified. The length of the contigs varied from 120 nt to 2,180 nt. Viruses identified in more than five wild boars are given in the table below (Table 3). Other

viruses identified in less than five wild boars were porcine astrovirus, porcine pestivirus, porcine hokovirus, swine influenza virus (H1/N1), porcine sapovirus, and the vector borne Schmalenberg virus. Sequences similar to tick borne encephalitits virus was identified in one wild boar. The final data is to be compiled and the manuscript is in preparation. The results so far identify a high risk for transmission of viruses between wild boars and livestock in the farms.

Table 3. Number of Swedish wild boars analyzed by NGS, and viruses identified in more than five wild boars are shown.

Age/virus	<1 year	1-2 year	adult	total
Number of wild boars	10	4	9	23
HEV: Negative	2	0	1	3
Anti-HEV positive	3	3	4	10
HEV RNA positive	5	1	4	10
Porcine circovirus	10	4	8	22 (96%)
Parvovirus[1]	10	4	6	20 (87%)
Sus Torque Teno virus	10	3	8	21(91%)
Equine infectious anemia virus	6	2	3	11 (47%)
Picornavirus[2]	10	4	8	19 (83%)
Porcine bocavirus	5	4	5	14 (61%)

1 The parvovirus sequence was similar to a virus identified in contaminated kits for nucleic acid extractions in China (Accession No. KM105951)

2 Three wild boars were infected with pasivirus, which is similar to parechovirus and has been isolated from domestic pigs in France. Five wild boars were infected with posavirus isolated form young piglets and in water from pig farms.

4.2 Paper II: Hepatitis E virus strains infecting Swedish domestic pigs are unique for each pig farm and remain in the farm for at least 2 years

Paper I evaluated the HEV epidemics in Spanish and Swedish wild boars and showed that zoonotic transmission from wild boars may be more common than previously anticipated. To better understand the HEV transmission between the animal reservoirs and humans, the HEV prevalence and genetic diversity in domestic pigs were investigated by collecting 363 fecal samples from 3-month old piglets sampled with more than one year apart from 30 Swedish pig farms as described in **Paper II**.

4.2.1 HEV RNA prevalence in Swedish pig farms and piglets

In this study, HEV RNA positive piglets were detected in 77% of 30 investigated Swedish pig farms. Samples were collected with more than one year apart from each farm. Eight farms had piglets excreting HEV at both sampling occasions. In total, HEV RNA was found in feces from 79 piglets (79/363; 22%; Table 4). There were significantly more farms with infected piglets in 2013 compared to 2014 ($p = 0.0033$). The reason for this may either be due to different sampling by the staff at the animal health, or that the prevalence of HEV is declining. The results from this study corroborates a previous study from Sweden in 2005, where 29.6% (71/240) of tested fecal samples from 16 of 22 pig farms (72.7%) were HEV RNA positive [159]. These results indicate that the HEV prevalence in Swedish pig farms has been stable during at least 8 years. However, there are approximately 1,100 pig farms in Sweden and around 2.6 million pigs are produced yearly. Only a small fraction of the farms has been investigated so far. It is therefore difficult to ascertain the HEV prevalence and its possible changes until more farms have been investigated. We have shown that strains from humans infected in Sweden are similar to HEV strains from pigs. Most of the pork produced in Sweden is consumed in the country, accounting for about 70% of all pork sold [157]. The presence of HEV infections may not be a big problem for pig farmers, since the infection in pigs is usually subclinical [172].

4.2.2 HEV RNA prevalence in different types of Swedish pig farms

Four different breading types of pig farms were investigated in this study. There was no difference between the type of farm and number of HEV-infected piglets (Table 4). However, there was a difference in number of HEV positive piglets in the organic farms at the two sampling occasions (21/54 in 2013 vs 4/54 in 2014). The reason for this is not clear, but may be due to differences in sampling.

Table 4. Type and number of farms and number of piglets investigated, and number of farms with HEV excreting piglets.

Type of farm	Number of farms	Number of fecal samples from piglets/year		Sub total	Number of farms with HEV RNA positive piglets/year		Total number of farms with positive piglets
		2013	2014		2013	2014	
Non-closed purchased gilt	7	42	42	*84*	6 (86%)	5 (71%)	7 (100%)
Closed	7	42	45	*85*	3 (43%)	3 (43%)	5 (71%)
Pool sharing sows	7	42	42	*84*	3 (43%)	3 (43%)	4 (57%)
Organic	9	54	54	*108*	5 (56%)	3 (43%)	7 (78%)
TOTAL	30	180	183	*363*	17 (57%)	14 (47%)	23 (77%)

In Sweden, nearly all pig production is indoors and almost all pigs are produced "batch-wise". The pig pens and houses are cleaned between each batch. It is thus an "all-in, all-out" system that prevents the spread of infectious agents between each batch or from elder to younger pigs. Less than 2% of the pigs are raised in ecological farms, where the piglets can be outdoors [157]. In these farms, the piglets may come in contact with wild animals, as wild boar, deer, moose and rat, which can be HEV infected [194, 220]. The HEV RNA prevalence in the Swedish wild boar population was 15% (Paper I), which is slightly lower than the prevalence in piglets raised in ecological pig farms (23%). The reason for this difference may partly be due to the living habits of two species or age difference between the two groups. The piglets may infect each other more frequently than the young wild boars, since they live in the same limited area. They may also excrete the virus into the soil and environmental water, from which new batches of piglets may become infected. With the continuous expanding of territory and population of wild boars in Sweden, the contacts between the two species may become more frequent than before. This may increase the risk of transmission of HEV and other viruses between the two species.

4.2.3 Phylogenetic analysis of HEV strains circulating in Swedish pig farms

Partial ORF1 could be amplified and sequenced in 46 of the 79 HEV RNA positive samples from 14 pig farms. All strains belonged to HEV3, and all but four were subtype HEV3f (Figure

7). The other four strains were from the same farm (Farm 12) and belonged to subtype HEV3e. These subtypes had been identified in Swedish pig farms previously [159]. Co-circulating of HEV3f and HEV3e in pig farms has also been reported from other European countries [219, 221].

Each pig farm had a unique HEV strain and the dominant strain remained in the farm between the two test occasions, and probably for longer periods. Only one farm (Farm 2) had obtained a new strain, which was similar to strains from wild boars. The HEV strains from each farm were not similar to strains from nearby pig farms. They were intermixed with strains from other counties. The reason for this is not known. Two farms in the county of Västmanland had similar strains that formed one clade in phylogenetic tree. This suggests that these strains have a common ancestor, and have recently diverged from each other.

In Sweden, after weaning from sows, some pig farms keep the piglets in the growing stables until slaughter, while others send the piglets to another fattening farms, where they live with other herds until slaughter. In this study all samples were collected from 3-month old piglets still living in the growing stables. The piglets may get the HEV infection in two ways, either from the sow or in the growing stables. The possibility of transmission from sows is low, since most of older pigs have anti-HEV antibodies and only a few are infectious [222]. In addition, some pig farms purchase gilts from other farms. If sows would be the main source of infection, the dominant strain in the farm should change with each new batch of gilts/sows. Also in the farms sharing sows, the sows are sent to different satellite farms, however, each farm still had its own unique strain despite the exchange of sows. This all indicates that the piglets are acquiring the infection in the farm during the period in growing stables. It is known that HEV can survive for longer periods in environments, as in wastewater and soil [223]. The infected piglets from previous batches may have excreted virus particles into the environment. The virus may be spread into soil and water wells through manure ditches, where HEV was detected [222, 224]. When a new batch of piglets enters the farm, the virus may thus spread through contact with contaminated materials, food, or water. Furthermore, the role of rodents in the transmission of HEV in pig farms is still under debate. Rodents, especially rats, live close to humans and domestic pigs, and recent studies have detected both rat HEV and HEV3 in wild rats [225, 226]. Even if experimental transmission failed to transmit rat HEV to pigs [227], the risk of transmission of HEV3 from rats to humans and pigs cannot be ruled out.

It was noted that although unique HEV3 strains remained in four of five pig farms, the strains in Farm 2 were found at two separate branches, with strains from 2013 on one branch and those from 2014 on another branch (Figure 7). Two possible reasons may lead to this. First there may be sampling bias, if multiple strains were circulating in the pig farm at the same time. However, all samples collected at each sampling occasion were had the same specific strain. Thus, we may have missed the other circulating strain, since only six piglets were sampled from each farm at each occasion. Another reason could be that the dominant HEV strain has changed between the samplings, especially since the new strain was similar to strains from wild boars. This wild boar strain may perhaps be more virulent that the previous dominant strain and has spread rapidly in the farm. Since this farm, Farm 2, is a non-closed farm purchasing gilts, the replacement of the dominant strain may have occurred by the introduction of an infected sow

from another farm.

Some wild boars and piglets from the same region were infected with similar strains, as the strains from Farm 2, indicating a zoonotic transmission, but transmission direction and route between two species still need to be investigated. It should be noted that the direct and indirect contact between domestic pigs and wild boars not only transmit HEV, but also other pathogens, such as porcine parvovirus, swine influenza virus, *Erysipelothrix rhusiopathiae*, *Mycoplasma hyopneumoniae*, and *Toxoplasma gondii* [228]. Several of these pathogens have been detected in Swedish wild boars (See Paper I), and some of them could cause severe damage to the pig industry or diseases in humans.

The HEV strains from domestic pigs were also genetically similar with strains from Swedish patients. Almost all infected humans had unknown source of infection. The high genetic similarity between the human and the pig strains suggest that the infection may have been through consumption of contaminated pork meat or products, or through direct contact with infected pigs. In Paper I, we showed that HEV3/i strains from chronic infected patients were on the same clade as a strain from a Spanish wild boar, although this subtype has not been observed in Swedish pigs or wild boars.

In this study we monitored a possible change in dominant HEV strains in 30 pig farms at two occasions two year apart. A long-term monitoring in the future is needed to better clarify the HEV epidemic in the pig farms and to determine how long HEV can survive in the environments during natural conditions. Such study will also indicate which factors change the dominant HEV strains in the farms. With this solved, the finding of unique HEV strain in each farm could be used to determine if HEV infections in humans derive from meat items that have been locally produced or imported from other countries. HEV typing may even identify the origin of the strain to pig farm level.
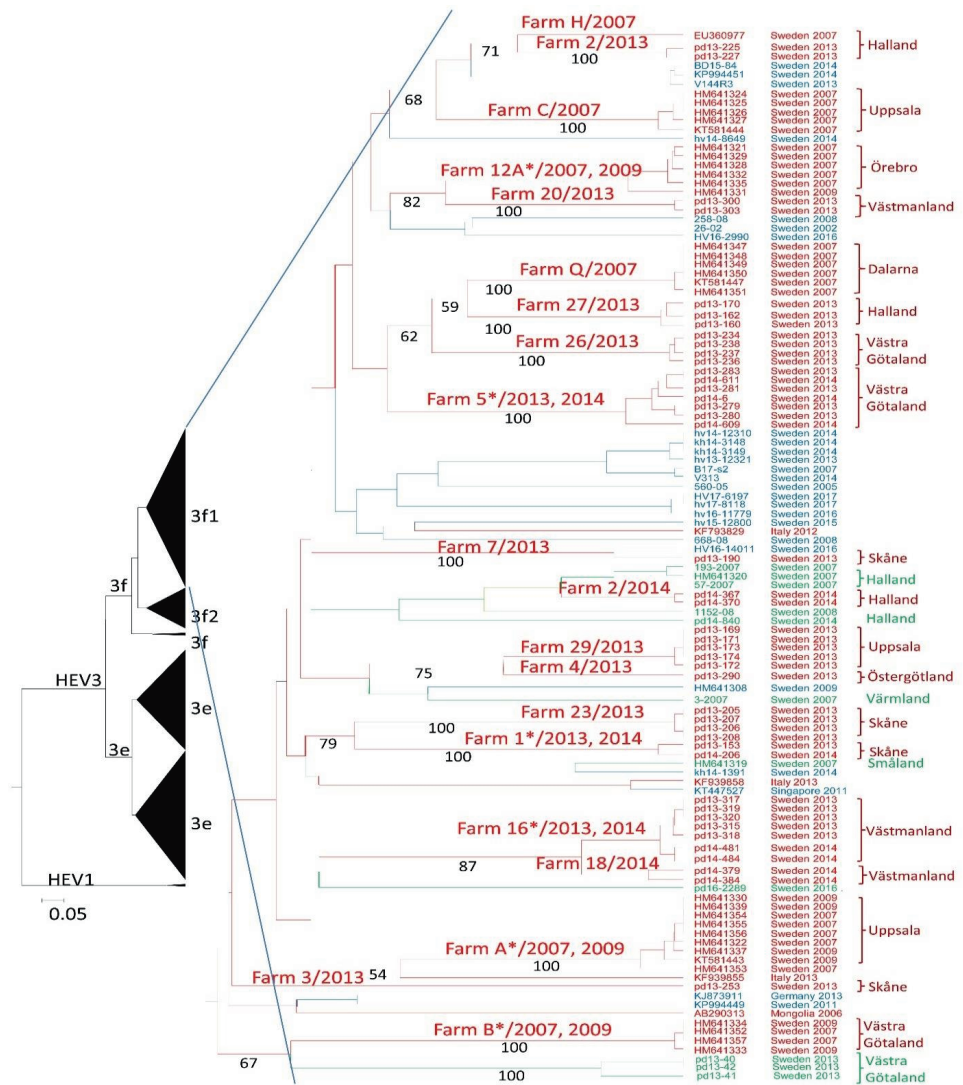
Figure 7. The enlarged branch of HEV3 subtype 3f strains in a UPGMA phylogenetic tree based on 325 nucleotides of partial ORF1 for genotype 3. The strains from pigs are given in red, those from wild boars in green and those from humans in blue. The farm designations where the strains were collected are given on the branches and the region of origin of the farms are given at the nodes. The farms in which samples were collected in 2007 and 2009 are indicated with year after the name of the farm. Farms marked with an asterisk indicate farms with strains collected more than a year apart.

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4.3 Paper III: Differential removal of human pathogenic viruses from sewage by conventional and ozone treatments

In **Paper I and II**, we explored the HEV prevalence in animal reservoirs, as wild boars and domestic pigs, and showed the risks of HEV transmission from animals to humans. The HEV-infected hosts may excrete virus particles into surface water or wastewater. Since the virus is stable in this environment, it may infect a new host by waterborne transmission. In **Paper III, IV, and V**, the presence of HEV, and 12 other common enteric viruses were investigated in samples from two WWTPs and two DWTPs. The viral diversity was also investigated in some of these samples and in tap water by NGS. The aim was to investigate if HEV and other human enteric viruses disseminate in the population through water. To achieve these goals, a universal and effective technique for concentrating viruses from larger amounts of multiple aquatic environments was needed. In **Paper III**, we developed a technique for concentration of viruses, which was applied to investigate the efficiency of virus elimination by conventional treatment and additional ozone treatment of the wastewater before its release from Knivsta WWTP.

4.3.1 Evaluation of a concentration method for viruses from water

Viruses are normally present at low concentrations in aquatic environments. Therefore, concentration of viruses from large volume of water is a prerequisite prior to downstream applications. In this study, a known amount of human adenovirus 2 (HAdV-2) was added to purified water and raw sewage. The virus was concentrated by our in-house developed method, which combines NanoCeram filtration and ultracentrifugation. The efficiency of virus concentration was evaluated by both qPCR and isolation of the virus on cell culture. The qPCR showed a recovery of 7% for adenovirus by this method. The isolation on cell culture showed that the viruses were viable after the concentration and had been concentrated 1,000 times.

The quantities of viruses vary greatly in different types of waters. The concentration of viruses is normally low in ground water, recreational water, and drinking water, and a large volume is required for recovery of the viruses. For wastewater, the virus concentration is relatively high, and a smaller volume is sufficient for detection of the viruses. NanoCeram Virus Sampler filter was used for concentration of viruses in water. It is an electropositive non-woven filter media in a pleated cartridge. This filter can retain viruses from multiple types of water. It can be used for filtering water with a high turbidity, as sewage, and also up to 50,000 liters of water with a low turbidity, as drinking water. This filter was recently recommended by the United States Environmental Protection Agency to replace 1MDS filter as the primary concentration method of enterovirus and norovirus in water [229, 230]. Some studies have shown high recovery of viruses in drinking water, surface water and sewage by using NanoCeram filters [231-233]. However, the recovery of adenovirus has been shown to be lower than for other viruses. Gibbons et al. reported a recovery over 96% for norovirus and male-specific coliphages, but less than 3% for adenovirus in natural seawater [234]. We assumed that if a satisfying recovery for adenovirus could be achieved, then the recovery of other viruses would be higher. Based

on this assumption, HAdV-2 was used as a viral indicator in the evaluation. The recovery of 7% was higher than that from several previous studies [234, 235]. Another reason to choose HAdV-2 to develop the technique is the possibility to grow the virus on cell cultures. The viability of the virus could thus be determined.

The elution of viruses from the filters turned out to be difficult, and needed several experiments until satisfying results were obtained. The elution volume from NanoCeram filter was about 330 mL. Therefore, a second concentration method was needed to further reduce the volume before downstream applications. Many different techniques, such as organic flocculation, precipitation, ultrafiltration, and ultracentrifugation, have been used as an secondary concentration method for viruses from water [236]. We analyzed PEG precipitation, Amicon stirred cells, and ultracentrifugation, and found that ultracentrifugation had the highest recovery for HAdV-2. It was therefore used as the second concentration method in our in-house developed method.

The number of viruses in aquatic samples is very low, and their genome size are much smaller than that of other microorganisms in water, as bacteria, archaea, and eukaryotes. For the identification of viruses by molecular techniques, there is therefore a need to remove genomes from other organisms in order to improve the proportion of virus sequences. Free genomes were therefore degraded in the concentrated water samples with Benzonase nuclease, an enzyme that can degrade all forms of free DNA and RNA while it has no proteolytic activity. This treatment did not affect the concentration of HAdV-2 determined by qPCR, and was therefore used in subsequent analyses.

4.3.2   Virome in raw sewage

Three incoming sewage samples were sequenced on the Ion Torrent platform. A total of 309,881 to 444,559 reads were obtained after quality control. Between 350 and 2,900 reads or contigs were identified as virus sequences, which accounted for 4.1% to 6.4% of all blasted sequences. A high diversity of viruses in raw sewage was revealed by the analysis. There were in total 327 different virus species belonging to 25 known virus families identified in the samples.

The identified viruses could infect bacteria, protists, plants, vertebrates, and invertebrates. Bacteriophages accounted for the largest proportion, and mainly belonged to the *Inoviridae*, *Microviridae*, *Myoviridae*, *Podoviridae*, and *Siphoviridae* families. Sequences similar to gokushovirus, a member of the *Microviridae*, was abundant in all samples. Gokushovirus has a worldwide distribution. It has been found in human gut, animals, soil, and volcanic sediment [237-239]. It is also prevalent in many aquatic environments, such as freshwater, marine, and spring surface water [240-242]. The wide abundance and spread of gokushovirus suggest its potential as a virus indicator in water research, which will be evaluated in the next two studies.

Plant viruses were also abundant in raw sewage. One representative is pepper mild mottle virus (PMMoV). This virus was originally isolated in pepper [243]. It was found at high concentrations in human feces after consumption of pepper products and has been used as an

indicator of fecal pollution [244]. Subsequent studies frequently detected PMMoV in multiple aquatic environments, including drinking water sources. It was shown to have less seasonality than human enteric viruses. Its source was most likely from human feces, and it was therefore recently proposed as an indicator of human viruses in the assessment of water treatment and quality [136, 245].

Human viruses were also found in the sewage, most of them were associated with gastroenteritis. The most common viruses were HAdV-41, human astrovirus 1 and 4, parvovirus, human picobirnavirus, and human feces pecovirus. Among them, HAdV and astrovirus are routinely diagnosed in patients with gastroenteritis, whereas the others are not. Besides enteric viruses, there were also sequences similar to human papillomavirus (HPV), two belonged to the *Betapapillomavirus* genus and the other to the *Mupapillomavirus* genus. HPV is mainly spread through sexual contact and could cause warts, papilloma, and malignant tumors. Waterborne transmission has also been suspected. Several types, including the oncogenic high risk genotype HPV16 and 18, have been detected in urban sewage worldwide in recent years [132, 137, 246, 247], which may partly explain HPV infections in persons who had never been sexually active [137]. Further studies to confirm this route, as well as to understand if HPV is resistant to the inactivating treatments used for wastewater are still needed.

In this analysis, the identification of viruses was based on BLAST scores. Apart from the identified reads, there were plenty of reads and assembled contigs that failed to satisfy the identification criteria. Some of them could be caused by inaccurate assembly producing artificial chimeric genomes. Others had some similarities to known viruses and may be potential novel viruses. For these contigs, there were no high similarity to reference sequences in GenBank. However, they showed partial similarity to sequences of viruses belonging to the same virus family. This may indicate that these possible novel viruses may originate from known virus families, but with only somewhat similar sequences. Furthermore, a large proportion of the assembled sequences showed no or little relation to any known sequence in the current genomic database. Some of these sequences may represent uncharacterized viruses. Further examinations with other methods are needed to confirm possible presence of novel viruses.

4.3.3 The removal of viruses by conventional and ozone treatments

Primers and probes for seven selected viruses were designed based on the NGS results. These seven viruses together with 14 common enteric viruses were tested in influent sewage, conventional treated sewage, ozone treated effluents, and after passing an open dam system. All seven NGS-selected viruses were detected in influent sewage by qPCR, which validated the NGS results. Among the 14 common enteric viruses, seven viruses were detected in raw sewage (Table 5). All of them, except for HPeV and HEV are common causes for diarrhea and are routinely tested for at hospital laboratories. This indicates that the detection of enteric viruses in sewage could reflect the circulation of viruses in local communities.

The conventional treatment of sewage reduced most of the tested viruses by one to four log10.

Norovirus GI, sapovirus, HPeV and HEV were reduced to undetectable levels (Table 5). The concentrations of most viruses were lowered with 98.9% to 100%, however, adenoviruses were less affected (62% to 96%). The removal of NGS-selected viruses showed diverse efficiency at the WWTP. The reduction of gokushovirus was higher than 99.97% during three sampling periods. The reduction of parvovirus and parvovirus-like virus were only 0% and 25% during week 51/52, and 53% and 49% during week 50. These viruses were reduced to more than 99% in week 49. It seems as if conventional treatment was less efficient on DNA viruses than RNA viruses. However, the bacteriophages, like gokushovirus, are ssDNA virus, and they were efficiently reduced. The reduction may depend on differences of physiochemical properties for each virus. Further studies are needed to illustrate which factors may affect the viral removal efficiency.

An ozone dose of around 6 mg/L was added to the effluent sewage. This reduced the amount of viruses to undetectable levels for astrovirus, pecovirus, picobirnavirus, parvovirus-like virus, and gokushovirus. For other viruses, the concentrations were lowered by between 85% to 100% in the different samples. It was less efficient for adenovirus, where only 55% to 91% removal was achieved. Ozone is an extremely reactive oxidant. The oxidation could change the conformation of viral capsid proteins, thereby damaging the capsids, or suppressing the virus/host cell receptor binding [248]. It has been used as a disinfectant for drinking water in Europe for a long time. However, the effectiveness to inactivate viruses in sewage may be hampered by high amounts of organic matter [249]. This matter may provide a physical protection by adhesion of the viruses to organic particles. Hence, removal of organic matter is necessary before ozone treatment to achieve satisfying reduction of viruses. Studies have showed that ozone treatment after conventional treatment of sewage is highly efficient in inactivating bacteria and bacteriophages [250]. It also effectively removes micro-contaminants, such as pharmaceuticals [251]. Here we found that ozone treatment for reducing human viruses in treated sewage may be a promising technology, which will simultaneously remove multiple hazardous materials before the release of the treated wastewater from the WWTPs.

The presence of 21 viruses were also tested in the water in a pond to which the water was led after the treatment and before it was released into the nearby river. The viruses detected in this water were HAdV, norovirus GII, astrovirus 4, parvovirus, parvovirus-like virus, and pecovirus. The amounts of these viruses were at least 92% less than in raw sewage. Several of these viruses were not detected after ozone treatment but re-appeared in the pond at low concentrations, as pecovirus, parvovirus, parvovirus-like virus, adenovirus 41, and astrovirus 4 in week 50 (Table 5). The reason for this is not clear. One possible explanation could be the presence of high loads of suspended solids in the water, which may compromise the effectiveness of the ozone disinfection. Some viruses may therefore have escaped the treatment. Also, about 4% of the conventionally treated wastewater did by-pass the ozone treatment and entered directly into the pond during week 49. This was due to partial clogging of the protective sieves for the lifting pumps in the ozonation step. Although the virus concentrations in the effluents were low, there is a risk that the viruses may infect new hosts coming in contact with the water. Continuous monitoring of the presence of human pathogenic viruses in sewage would be helpful to control such potential risks.

Table 5. Real-time PCR for seven common enteric viruses and seven viruses identified by NGS and the estimated number of genomes of these viruses/mL water in untreated and treated wastewater samples from three weeks in 2015, where K numbers indicate internal sample number.

Virus		Week 49				Week 50				Week 51/52			
		K399	K400	K401	K402	K416	K417	K418	K419	K462	K463	K464	K465
		Inlet sewage	Conventionally treated	Ozone treated	Outlet after dam	Inlet sewage	Conventionally treated	Ozone treated	Outlet after dam	Inlet sewage	Conventionally treated	Ozone treated	Outlet after dam
Adenovirus	Ct value	27.1#	34.7	30.6	36.0	33.0	34.4	35.6	36.7	32.1	36.9	40.3	39.7
	Viral genomes/mL	260,000	1,300	24,000	530	4,200	1,600	720	330	7,900	290	27	40
Noro GI	Ct value	30.0	-*	-	-	35.6	-	-	-	29.7	-	-	-
	Viral genomes/mL	36,000	-	-	-	730	-	-	-	43,000	-	-	-
Noro GII	Ct value	29.1	36.5	39.1	38.3	32.1	38.6	-	-	28.9	36.1	-	38.7
	Viral genomes/mL	65,000	390	61	11	8,400	88	-	-	76,000	50	-	80
Sapovirus	Ct value	26.6	-	-	-	31.4	-	-	-	27.1	-	-	-
	Viral genomes/mL	380,000	-	-	-	14,000	-	-	-	270,000	-	-	-
Parechovirus	Ct value	33.6	-	-	-	35.3	-	-	-	35.4	-	-	-
	Viral genomes/mL	2,800	-	-	-	850	-	-	-	780	-	-	-
HEV	Ct value	-	-	-	-	-	-	-	-	39.6	-	-	-
	Viral genomes/mL	-	-	-	-	-	-	-	-	45	-	-	-
Astrovirus	Ct value	28.8	-	-	-	25.3	-	-	-	25.0	37.5	-	-
	Viral genomes/mL	81,000	-	-	-	960,000	-	-	-	1,100,000	180	-	-

Pecovirus	Ct value	21.03	31.2	-	-	24.32	31.02	-	32.08	24.22	29	-	-
	Viral genomes/mL	18,000,000	15,000	-	-	1,900,000	17,000	-	8,200	2,000,000	71,000	-	-
Picobirnavirus	Ct value	27.73	31.16	-	-	28.05	32.87	-	-	27.17	30.94	-	-
	Viral genomes/mL	170,000	16,000	-	-	140,000	4,700	-	-	250,000	18,000	-	-
Parvovirus	Ct value	21.8	29.34	-	33.91	26.29	27.38	-	29.9	26.02	26.46	32.72	33.74
	Viral genomes/mL	11,000,000	56,000	-	2,300	470,000	220,000	-	38,000	560,000	420,000	5,300	2,600
Parvovirus-like virus	Ct value	20.3	26.92	-	-	25.11	26	-	28.6	25.04	25.02	-	-
	Viral genomes/mL	31,000,000	300,000	-	-	1,100,000	570,000	-	93,000	1,100,000	1,100,000	-	-
Gokushovirus	Ct value	23.29	34.89	-	-	20.51	-	-	-	20.11	31.78	-	-
	Viral genomes/mL	3,800,000	1,200	-	-	26,000,000	-	-	-	35,000,000	10,000	-	-
Astrovirus 4	Ct value	22.27	27.66	-	-	24.11	27.98	-	29.70	21.84	25.74	33.20	35.50
	Viral genomes/mL	7,700,000	180,000	-	-	2,100,000	140,000	-	43,000	10,000,000	680,000	3,800	760
Adenovirus 41	Ct value	29.49	35.56	-	-	-	37.94	-	37.26	35.1	37.68	37.23	-
	Viral genomes/mL	50,000	730	-	-	-	140	-	220	1,000	170	230	-

#: Mean Ct value (Ct=cycle threshold in the quantitative PCR)

*: Undetected

4.3.4 Phylogenetic analysis of adenoviruses

Our analysis showed that adenoviruses were less sensitive to conventional and ozone treatments than other viruses. Adenoviruses could be detected in all samples by qPCR. A nested PCR was designed to amplify its partial hexon gene for sequencing and typing. Nine strains could be sequenced. Multiple HAdV strains were detected. Six strains belonged to species F (five from HAdV-41, one from HAdV-40), and two strains to species A. The ninth strain from ozone treated water in week 49 could not be typed, but was found on the same branch as species F strains in the phylogenetic tree (Figure 8). Similar result was also reported in another study, where up to 20 different HAdV types were detected in sewage by HAdV targeted metagenomics [131]. The phylogenetic tree in this study showed that strains belonging to species F were dominating in raw sewage. Two strains were HAdV-41 (samples 399 and 462) and one was HAdV-40 (sample 416). The dominating strain changed with treatment, and strains belonging to species A appeared in the effluent. This suggests that the different types of HAdV have varying degrees of sensitivity to conventional and ozone treatments. Since the oxidation mainly targets the capsid proteins of the virus, there may be differences in the composition and structure of these proteins for each virus type, which may contribute to the different sensitivities. However, the infectivity of HAdV after ozonation is still unclear. The virus DNA may be detected by PCR even if the virus capsids are altered. Further studies are needed to select adenovirus types that can grow on cells lines to test the infectivity of the virus after ozonation.

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AB562586 15
AP012285 65
AP012302 67
DQ393829 49
FJ169625 53
FJ404771 22
FJ824826 28
JF799911 59
JN162671 62
JN162672 60
JN226752 25
JN226756 32
JN226757 47
JN226759 38
JN226761 42
JN226762 43
KF279629 23
AB448778 37
JN226751 24
JN226749 20
HQ883276 58
JN226753 27
AB448769 8
NC 012959 54
AY875648 46
EF153473 48
EF153474 26
HM770721 56
JN226746 10
JN226747 13
JN226754 26
JN226755 30
JN226758 33
JN226763 44
JN226764 45
JN935766 63
NC 010956 9
GQ384080 36
AB448774 19
EF121005 64
AC 000006 17
JN226760 39
JN226765 51
AC 000008 5
HQ003817 57
HQ413315 6
AC 000017 1
NC 001405 2
DQ923122 52
AB728839 41
HM565136 41
KF303069 41
KF303071 41
KY316161 41
399-incoming sewage week49 41
AJ617298 41
JX412891 41
DQ315364 41
400-purified sewage week49
417-purified sewage week50
KU641149 41
462-incoming sewage week51/52
464-ozone treated week51/52
KC953636 41
AM920721 41
JX412899 40
KU162869 40
NC 001454 40
HQ268786 40
416-incoming sewage week50
401-ozone treated week49
463-purified sewage week51/52
AB330112 31
AM749299 31
JF964962 61
AY220987 31
419-outlet water week50
AB330093 12
AC 000005 12
KY859432 12
KY883427 12
GU191019 18
JN860678 68
JX423380 3
AY601636 16
AC 000018 7
AY337252 4
NC 003266 4
AY737798 50
KF528688 21
AC 000019 35
AY598970 11
AY803294 14
AY737797 34
KY780931 55
NC 029902

D

C

G
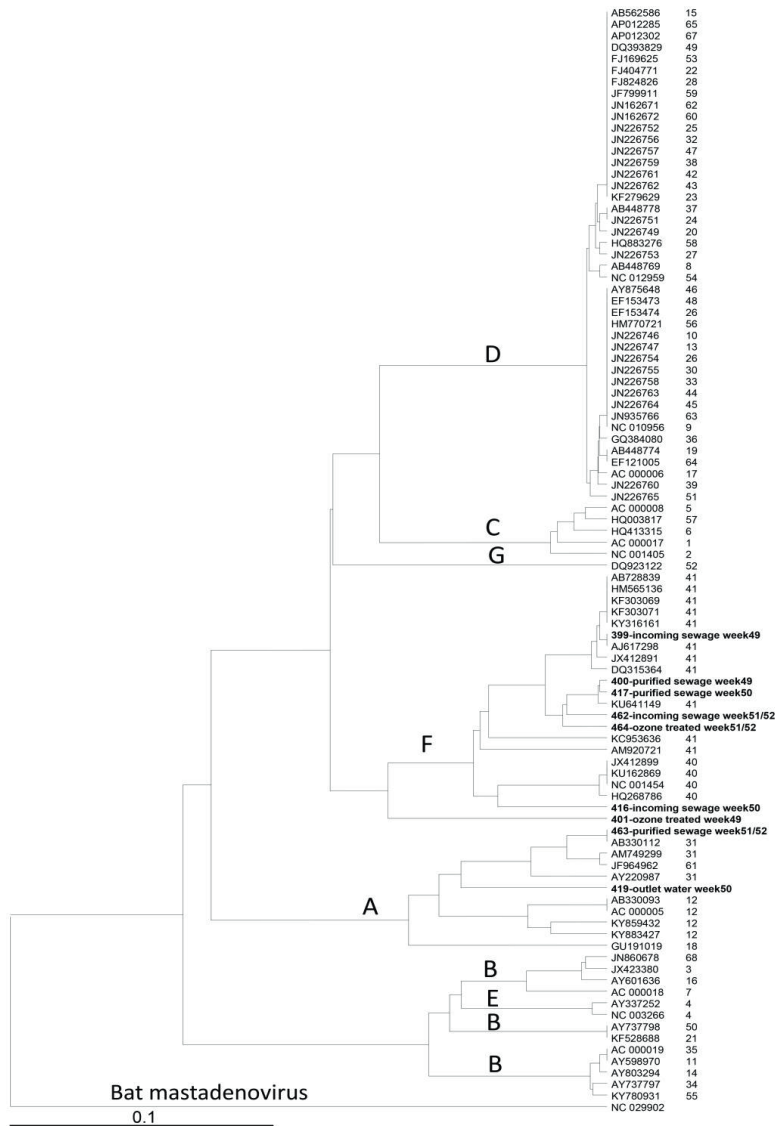
F

A

B

E

B

B

Bat mastadenovirus

0.1

Figure 8. Phylogenetic tree based on 185 nucleotides of hexon protein-coding region of adenovirus genome. Nine sequences from water samples and 81 adenovirus sequences representing all human mastadenovirus types from GenBank are included, and water samples are labelled in bold. The tree shows the dominant strains changed during the sewage treatment.

4.4 Paper IV: Hepatitis E virus genotype 3 strains and a plethora of other viruses detected in raw and still in tap water

In **Paper IV**, viruses were concentrated from water from different purification steps at two Swedish drinking water treatment plants (DWTPs), and in tap water by our in-house developed method, as described and evaluated in **Paper III**. The virome in the water samples was explored with NGS. The efficiency to completely or partially remove the viruses was compared at two DWTPs, one using UV treatment, the other using ultrafiltration as disinfection after conventional treatments of the raw water.

4.4.1 Virome in water from different purification steps and in tap water

To produce more virus reads, we switched from the PGM Ion Torrent used in Paper III to the Illumina HiSeq platform. A total of 8.5×10^6 to 31×10^6 raw reads per sample were obtained. The previous pipeline for identification of viruses was based on BLAST of the reads directly to GenBank non-redundant nucleotide database. This would have taken extremely long time, if we would have used that approach in this study, since 10-100 times more data was produced. Here we optimized the analysis workflow to accelerate the processes. All contigs and long single reads were first used for BLAST analysis to a database with all known virus sequences. This step identified "possible" virus reads in a short period. However, a larger proportion of them were probably not true virus reads. To reduce possible false-positive virus reads, a second blast was applied with the assumed virus read against to the GenBank non-redundant nucleotide database. This removed 75-97% of "possible" virus reads from the first blast analysis. For example, the first blast for raw water identify 62 different virus families, whereas only 26 virus families were found in the second blast. This classified 18% of the "possible" virus reads as significant virus reads. After this analysis, a total of 7.5×10^3 to 435×10^3 contigs or reads were classified as virus reads, accounting for 0.02% – 3.54% of all reads in eight samples (Table 6).

There were 1,374,050 virus reads per L raw water from Lackarebäck DWTP. This was the highest concentration of all samples. This sample also had the highest percentage of viruses (3.54% of all reads). The raw water from Lackarebäck DWTP is first treated by conventional carbon filtration. This treatment lowered the concentration of the viruses to 7,839 virus reads per L (Table 6). This concentration was similar to that in Alelyckan DWTP (2,488 virus reads/L), where also carbon filtration is used as first treatment. The carbon filtrations did not reduce the diversity of the viruses, but reduced the virus concentration with more than 2 log10. This efficiency of removing viruses is close to that found for some virus indicators, as the bacteriophages GA and PRD-1, and the PMMoV. It was, however, lower than for the bacteriophages MS2 and Qβ, which have more than 4.8-log reduction [252-254]. Since different viruses have distinct sensitivities to the different treatments, and the quality of the incoming raw water varies, the efficiency to remove viruses may vary with time and virus type, also for each virus indicator.

Table 6. Number of total reads of sequences and those corresponding to virus genomes obtained by NGS in the different water samples, not considering the recovery efficiency of viruses by the concentration method used.

Sample	Total reads	No. of identified virus families	Virus reads	Percentage virus reads	Virus reads/L original sample
Raw water (Lackarebäck)	8,544,764	26	302,291	3.54%	1,374,050
After carbon filtration (Lackarebäck)	28,075,226	26	435,500	1.55%	7,839
Permeate after UF (Lackarebäck)	23,766,188	16	7,574	0.03%	67
After carbon filtration (Alelyckan)	26,492,646	28	164,261	0.62%	2,488
After UV treatment (Alelyckan)	20,622,742	23	71,535	0.35%	916
Tap water - D1	25,895,686	18	23,688	0.09%	1,508
Tap water - D2	29,825,506	13	6,942	0.02%	540
Tap water - D3	31,253,164	12	33,633	0.11%	2,211

After conventional treatments, the pre-treated water passed through UF membranes to further remove pathogens in Lackarebäck DWTP. This step lowered the virus concentrations from 7,839 to 67 virus reads per L, which means an additional 2.1 log10 reduction of viruses. The number of identified virus families was reduced from 26 to 16. In Alelyckan DWTP, UV irradiation was used as an extra microbial barrier. This treatment lowered the concentration of the viruses from 2,488 to 916 virus reads per L, which was less efficient than UF. This difference may be caused by the different mechanisms of each method. As mentioned previously, UV irradiation mainly targets the confirmation of the viral capsid proteins, and may also attack the viral nucleic acids. However, the inactivation efficiency is dependent of the UV dose, and some other factors, such as the presence of residual particles and organic materials remaining from previous treatment steps may protect the viruses [255, 256]. The viability of the viruses after UV treatment was not determined. The efficiency of virus removal may thus have been higher than assumed in this study. In Lackarebäck UF replaced the UV disinfection. The pores of the membranes have a nominal size of 20 nm, which should normally block pathogens beyond this size. Thus, many of the viruses would be removed since they are larger than 20 nm. After treatment of the water with either UF or UV at two DWTPs, chlorine and chlorine dioxide are added before the water is pumped it into the supply network. We sampled

tap water for three consecutive days. The concentration of the viruses in these samples ranged from 540 to 2,211 virus reads per L, corresponding to 0.02–0.11% of the total reads. Although combined conventional and additional disinfection barriers achieved more than 4 log10 reduction of viruses at two DWTPs, there were still some viruses that passed through the treatment plant and entered into the supply network. This raises concerns for drinking water producers who do not practice the multi-barrier approach for drinking water recommended by the WHO [257].

Sequences homologous to viruses belonging to 12–28 known virus families and to about 650 non-classified viruses were identified in all eight samples. However, the composition of viruses changed with treatment. In raw water and water after carbon filtration at the two DWTPs, the most abundant sequences were similar to bacteriophages from the family *Microviridae*, which accounted for 35.3-57.4% of all virus reads. Their percentages were lowered to 11.1-15.0% after further disinfection with UF or UV, and were only 4.1% in one tap water. This indicates that viruses in this family have high sensitivity to the treatments. Another example is viruses in the *Mimiviridae* family. They are a group of giant viruses, and were present in high concentrations in raw water (1,086 virus reads/L; 7.2% of all virus reads). The carbon filtration treatment lowered their concentration to 1.3-2.3 virus reads/L. In Lackarebäck DWTP, the UF membranes removed these viruses to undetectable level, while the UV in Alelyckan reduced 90% of them. In addition, these viruses were not detected in two tap water. On the contrary, viruses in the *Virgaviridae* family were relatively stable with 80–240 reads in all samples except in one tap water sample. One representative from this family is PMMoV. We have discussed its usage as indicator of viruses in water in Paper III. The viruses from the *Virgaviridae* family are rod shaped particles about 20 nm in diameter and up to 300 nm long [258], This morphological feature may make it more easily for them to pass through the 20nm size UF, than other viruses of the same size, but with cubic symmetry of their capsids. The most common viruses infecting vertebrates identified in the water belonged to the *Hepeviridae*, *Parvoviridae* and *Circoviridae* families. Among them, sequences homologous to HEV from the *Hepeviridae* family were abundant and were selected for further analysis.

4.4.2   Detection and phylogenetic analysis of HEV in water samples

In raw water, the concentration for HEV was 387 reads per liter, and accounted for 2.56% of all virus reads. The number of HEV sequences per L water was reduced with about 3.6 log10 reduction in Lackarebäck DWTP, and 3.0 log10 reduction in Alelyckan DWTP. Although a high removal of HEV was achieved, still 1.7-20.0 HEV reads per L were identified in tap water. To further confirm this finding, the HEV genomes were quantified by qPCR. The amount of these genomes was calculated based on the on the regression line obtained from log10-dilution vs Ct values of the dilutions of the WHO standard (6329/10), assuming 20% recovery of viruses during concentration by Nano-Ceram filtration. HEV RNA could be quantified from six water samples except in raw water and water before UV treatment at Alelyckan DWTP. The amount of HEV genomes ranged from 6 to 130 IU HEV RNA per mL, with the lowest amount in water after UF at Lackarebäck DWTP and the highest amount in one tap water. The analyzing methods used in this study do not distinguish between infectious and non-infectious viruses.

Therefore, the infectivity of the viruses found in the water is unknown, and further studies on their viability are needed. Although HEV was detected in tap water, its amount per liter was comparably low. However, since the lowest infection dose for HEV is unclear, the risk of HEV infection by direct drinking tap water should be further evaluated.

In developing countries in Asia and Africa, the transmission of HEV is usually waterborne and caused by HEV1 and HEV2. In Europe, HEV transmissions are mainly zoonotic, and often associated with HEV3. Several studies have detected HEV3 in seafood grown in coastal waters [46, 259], indicating that HEV3 is prevalent in coastal waters. Consensus sequences of 820 nucleotides in the junction region of ORF1-ORF2-ORF3 could be obtained from all eight HEV positive samples based on the NGS analysis in this study. The phylogenetic analysis showed that one strain from the water after carbon filtration in Alelyckan DWTP belonged to subtype HEV3a, and was closely related to strains isolated from Canadian and South Korean swine (Figure 9). This subtype has been isolated from pigs in several European countries [31, 177, 260], and has rarely been isolated from Swedish piglets or wild boars. This subtype may be more common in Sweden than has been anticipated. Seven strains were shown to belong to subtype HEV3c/i. Three strains from tap water, two strains from Lackarebäck DWTP (before and after UF) and one strain after UV treatment from Alelyckan DWTP formed a separate branch within subtype 3c/i (Figure 9). The seventh 3c/i strain was from raw water, and was genetically similar to a strain isolated from a Swedish blood donor and to a strain isolated from sewage as shown in Paper V. This subtype is, as previously mentioned, often isolated from patients with chronic HEV in Sweden. Strains of subtype 3c/i were dominant in most samples. Whether this subtype is more easily spread through water than other subtypes is still unknown, and needs further evaluation. One recent study from France showed that persons drinking bottled water had a lower rate of anti-HEV IgG compared to those drinking tap water. This may indicate that contaminated water could contribute to the epidemiology of HEV infections [261]. However, there has until now not been any direct detection of HEV3 in drinking water. The results from this study suggest that HEV3 may also have waterborne transmission.

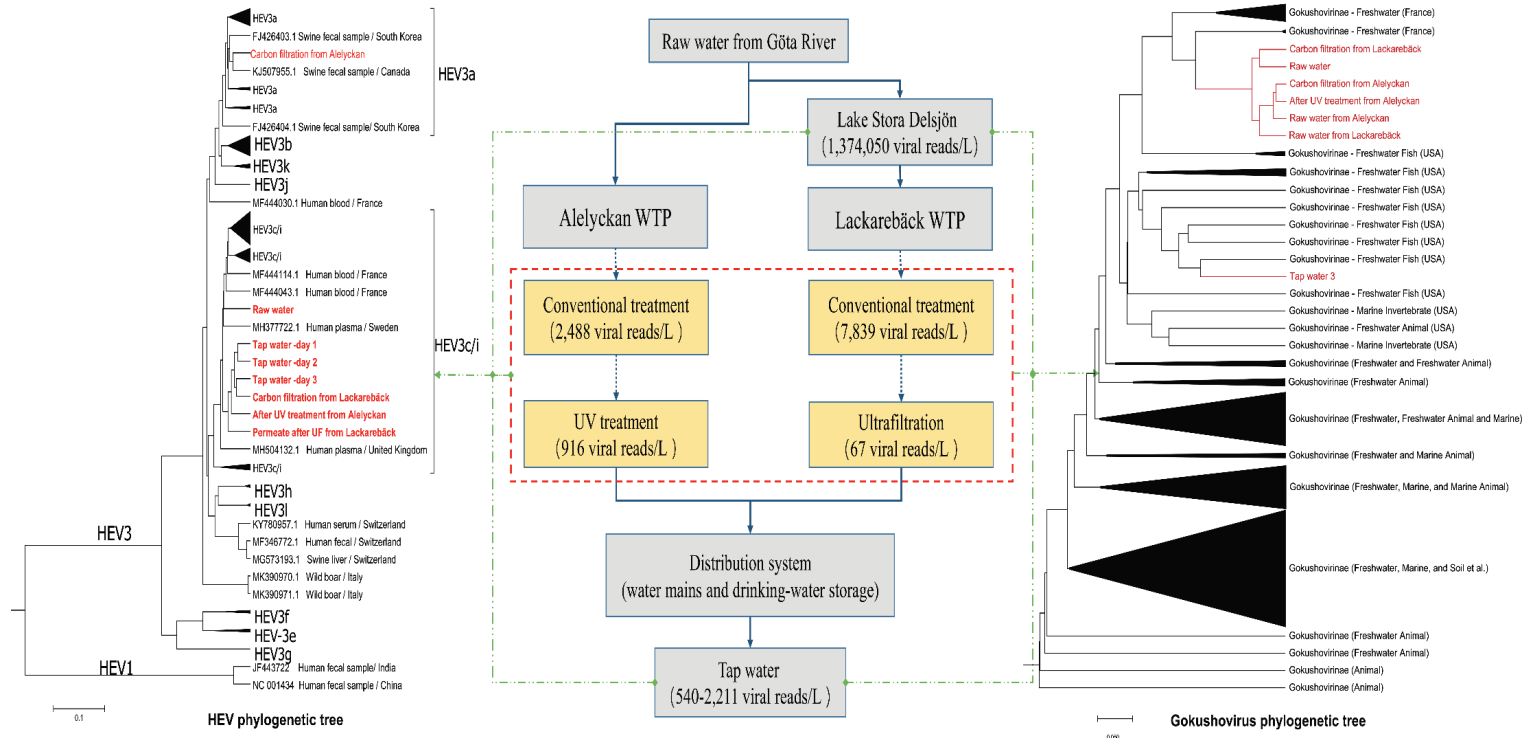*Figure 9. The different treatment steps at the investigated DWTPs, and the detection of HEV and gokushoviruses. Phylogenetic tree of 820 nucleotides of the junction region of ORF1-ORF2-ORF3 in HEV3 strains with HEV1 as outgroup and 357 nucleotides of partial VP1 in gokushoviruses. The strains from this study are marked in red. Accession number and origin of the strains are given at the nodes.*

4.4.3 Phylogenetic analysis of bacteriophages belonging to *Microviridae*

In Paper III, we found that sequences homologous to gokushovirus were abundant in all wastewater samples from Knivsta WWTP. Here we had similar results from two investigated DWTPs as previously mentioned. To validate the NGS results and to understand the phylogenetic relationships between the viruses in different types of water, a semi-nested PCR targeting partial VP1 of gokushoviruses within the *Microviridae* family was developed. Sequences were successfully obtained from seven samples. Three were from raw water, and one each from after carbon filtration in Lackarebäck DWTP, before and after UV treatment in Alelyckan DWTP, and in tap water. These seven strains together with 204 reference sequences belonging to the *Microviridae* family from the NCBI database were analyzed phylogenetically. The result showed all strains from this study were classified into the subfamily *Gokushovirinae*. Six strains from raw water and treated water in both DWTPs formed one clade on the same branch together with strains isolated from freshwater in France (Figure 9). The seventh strains from tap water was divergent from the other six strains. It formed another clade with strains from freshwater fish in the USA. The detection of a high amount of gokushovirus not only in WWTPs, but also in DWTPs from different parts of Sweden suggest that they could be good indicator of viruses for water microbial surveillance and evaluation of the treatments of water, at least in Sweden. The strain in tap water was genetically different from the other strains. The possibility cannot be ruled out that contaminations might be introduced into supply network during transportation or storage of the water. The water released from DWTPs goes through a long-distance transportation and has an uncertainty storage time before it is released in the tap. Although the frequency is low, several previous reports have shown that drinking water was contaminated due to the leakage of the water pipes, or contamination in the reserve water towers, or short intervals of negative pressure [150, 262-264]. Further investigations and comparisons of indicator viruses isolated from local water transportation pipes and storage facilities will help us to understand the role of the distribution networks in dissemination of viruses to humans.

4.5 Paper V: One year seasonal variations of enteric viruses in incoming and treated water at a wastewater plant

In **Paper IV**, the virome in water from different treatment steps at two DWTPs and tap water in the Gothenburg area was explored. In **Paper V**, the presence of 13 common human enteric viruses in influent and treated effluent wastewater at Rya WWTP in Gothenburg was investigated monthly during 2017. The virome in the treated effluents was also investigated by NGS. Seasonal variations of viruses in the wastewater was observed. The number of estimated persons infected with these viruses was calculated based on the viral genomes in raw wastewater. This number of estimated infected persons was compared to the number of diagnosed patients with these infections in Gothenburg area. The knowledge from this study will increase our understanding on transmission of viruses between humans and environments and give an estimate of the fraction of persons with subclinical infections caused by the various viruses. These results will together with the results from the drinking water study give us an overall picture of which viruses can be found in different water within Gothenburg area.

4.5.1 The prevalence and seasonal variation of common enteric viruses in influent wastewater

The presence of 13 common enteric viruses was monitored in influent wastewater from Rya WWTP during 2017 by qPCR. Eleven viruses were detected throughout the year. Among those detected, norovirus GII had the highest prevalence in all 26 samples, followed by astrovirus (25/26) and sapovirus (24/26). The lowest detection rate was for adenovirus, which was only found in 14 sewage samples. The estimated number of viral genomes for the 11 viruses detected was calculated based on the qPCR results (Table 7). The highest amount was observed for norovirus GI, with about 2.09×10^9 virus genomes per L wastewater. The lowest amount was for HPeV, which was present at a concentration of 6.90×10^3 virus genome copies per L in one sewage sample. The concentration for each virus varied in the samples during the year. It ranged from about 50 times (for aichi virus, HPeV, and enterovirus) up to 11,000 times (for norovirus GII).

The presence of human enteric viruses in wastewater is a reflection of the circulation of these viruses in the communities. Most of these enteric viruses identified in wastewater are currently routinely diagnosed in samples from patients with gastroenteritis or hepatitis at Swedish hospital laboratories. However, aichi virus and HPeV are not diagnosed routinely but were detected in the incoming wastewater. This suggests that there is a proportion of humans who are infected with these viruses. The prevalence and pathogenicity of these two viruses in the Swedish general population is thus unclear. However, the seasonality observed for these viruses may reflect yearly changes in number of infected. Aichi virus is shown to have a high seroprevalence in general population but with a low incidence [265, 266]. This virus has previously been detected in wastewater and surface water, and has been shown to cause

gastroenteritis in several countries. There is a high seroprevalence against HPeV in infants and young children in several European countries [267-269], but their prevalence in the Swedish general population is still unknown. The finding of a high amount of different human viruses in the wastewater in this study suggests that there are a number of infected persons excreting these viruses in Sweden, and that their prevalence in the general population may be higher than previously anticipated. There may, in addition, be other viruses found in the wastewater that have clinical relevance, as different astrovirus. The diagnoses in the hospital laboratories focus on the classical astrovirus groups. There are now two newly identified astrovirus groups, MLB-HAstVs and VA/MHO-HAstVs, which may not be detected with the current assays. These viruses were isolated in previous undiagnosed children with gastroenteritis symptoms [270]. It is debated nowadays if newly identified genotypes of some of the enteric viruses should be included in the routine diagnostics of samples from patients with gastroenteritis. Such diagnostic may be important for vulnerable populations, as young children or elderly, with gastroenteritis.

Table 7. Number of incoming effluent samples reactive for respective virus, and amount of viral genomes identified in the samples in relation to number of patients diagnosed with infection of respective virus during 2017.

Virus	Prevalence N reactive samples/N analyzed samples	Minimum estimated viral genome copies/L	Maximum estimated viral genome copies/L	N diagnosed patients infected with indicated virus[1]
Norovirus GI	19/26	2.69E+06	2.09E+09	21
Norovirus GII	26/26	1.52E+04	1.78E+08	418
Norovirus GIV	19/26	4.63E+05	7.26E+07	3
Adenovirus	14/26	1.56E+05	6.58E+08	172
HAV[2]	18/26	1.48E+04	1.93E+06	16
Rotavirus	23/26	6.97E+04	5.25E+07	99
Astrovirus	25/26	1.04E+04	8.09E+07	32
Sapovirus	24/26	3.24E+06	2.06E+09	69
Aichi virus	23/26	4.98E+06	2.42E+08	/
Parechovirus	19/26	6.90E+03	2.85E+05	/
Enterovirus[3]	18/26	3.99E+06	1.71E+08	/

1. There were in total 2,846 patients who had sought medical help for gastroenteritis symptoms during 2017. Their samples were diagnosed for enteric viruses at CML during 2017.

2. HAV RNA was analyzed in 32 samples from patients with anti-HAV IgM at CML during 2017. Only 16 of these reported cases of hepatitis A were living in the Gothenburg area when infected.

3. Enterovirus RNA was investigated in samples mainly from patients with suspected aseptic meningitis or upper respiratory infections at CML.
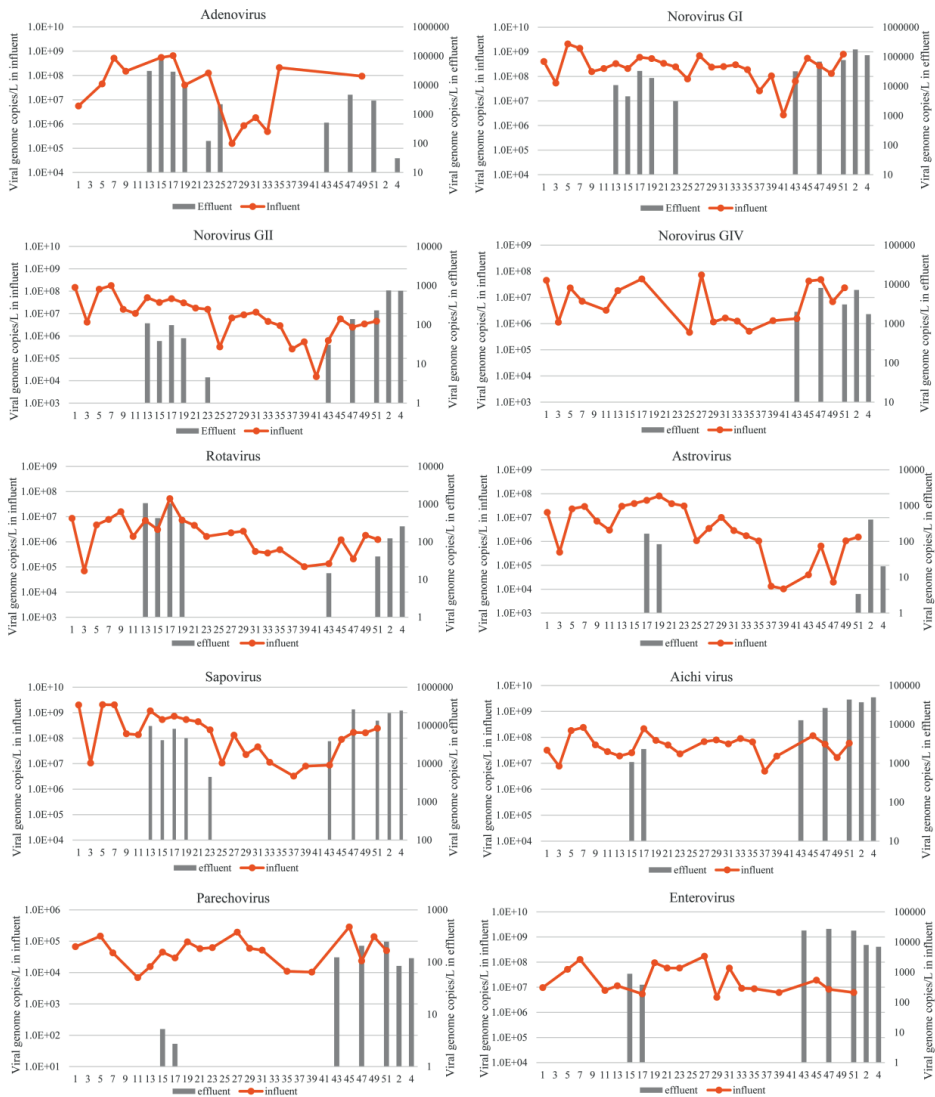
Figure 10. The number of virus genomes per liter influent and effluent wastewater between week 1 to week 51 during 2017 at the Rya WWTP. The quantification was performed by qPCR.

Two patterns of seasonal variations of the virus concentrations in the incoming wastewater were observed. One pattern was observed for aichi virus, HPeV, enterovirus, and norovirus GI (Figure 10). The concentration of these viruses was relatively stable during the year with few seasonal variations. Another pattern was observed for norovirus GII and GIV, astrovirus, rotavirus and sapovirus (Figure 10). For these viruses, their concentrations in the incoming wastewater was high during winter, and declined during summer. Thereafter their concentrations returned to high levels when winter was approaching. Seasonal patterns for

adenovirus and HAV could not be detected, since they had an uneven distributions and low detection rates. Enteric viruses in wastewater are excreted by the infected hosts, as humans and animals. Thus, seasonal changes in virus concentration in wastewater may reflect their seasonal distribution in the communities. For example, many studies have shown that norovirus and rotavirus infections have a seasonal trends with most infections during winter [271-273]. These seasonal profiles in wastewater paralleled with that in number of infected patients [274-276]. It should be noted that the seasonality of each virus in wastewater is affected by its viability. Many viruses are sensible to changes in the environment, as temperature, solar light, the concentration of organic matters, and the presence of indigenous microbial populations [277]. Further work on the viability of each human virus in water would be helpful to better predict their trends in human infections and wastewater.

4.5.2 Number of diagnosed patients with gastroenteritis compared to number of estimated infected persons

Patients with gastroenteritis in Västra Götaland region are routinely tested for agents causing the infection, as viruses, bacteria and parasites. There were 765 out of 2,846 routine samples reactive for gastroenteritis viruses at CML at Sahlgrenska University Hospital during 2017 (Table 7). The viruses identified were norovirus GI, GII, and GIV, sapovirus, rotavirus, adenovirus, and astrovirus. Most patients were infected with norovirus GII, which accounted for 58% (418/718) of all cases. Most of the infected (312/418) were detected between week 1 and week 20 of 2017. The second most common viral pathogen was adenovirus, which was identified in roughly 23% of the samples from the patients infected with gastroenteritis viruses. It had a wide distribution and was diagnosed weekly throughout the year. The infections caused by norovirus GI and GIV were relative rare, with only 21 and 3 diagnosed patients, respectively. There were between 32 and 99 patients diagnosed with astrovirus, sapovirus, or rotavirus (Table 7). They had similar seasonal patterns, with most cases diagnosed between week 1 and week 23. This trend with most cases during winter, paralleled the seasonal observation of norovirus GII, astrovirus, sapovirus, and rotavirus in wastewater.

Since most infections were caused by norovirus GII, and it also had the highest prevalence in incoming wastewater, thus it was used as an example to understand the difference between the number of diagnosed patients and estimated infected persons. It was shown to be a large difference between these two indicators, with more than 300 times more estimated infected persons than diagnosed patients during some periods. Although the difference between the two indicators was high, they showed similar trends during the year. The number of diagnosed and estimated persons was higher during winter and declined during summer. The difference between estimated and diagnosed patients may be due to a large number of infected persons having mild disease and do not seek for medical help. Regardless of symptoms, all infected persons excrete virus particles into the wastewater, which will end up in the WWTPs. There were three peaks for both estimated infected persons (at week 4-6, 12, and 16) and diagnosed patients (at week 3, 11, and 17-19) during the winter. It was roughly 1 to 4-weeks difference between the peaks from the two groups. Either the peak with viruses from the non-diagnosed

patients preceded the peak of diagnosed patients with one to four weeks, or the other way around. The reason for the high detection of norovirus GII during the cold season, may be due to the viability of this virus, which is largely affected by the temperature [278]. Temperatures below 4°C has been shown to be significantly associated with norovirus outbreaks [279]. Therefore, with rising temperatures and declining rainfall, the ability of norovirus GII to survive and transmit could be hampered. The time difference between peaks could provide a basis for the viral monitoring in sewage as an early warning system for viral outbreak in the future, still, further studies are needed to understand what factors affect viral transmission between humans and environments. This may facilitate more accurate predictions of upcoming outbreaks.

4.5.3 The removal efficiency of enteric viruses at Rya WWTP

The presence of the 13 enteric viruses in treated effluent wastewater was compared with its concentration in incoming wastewater. All 11 viruses identified in incoming wastewater were detected in treated effluent wastewater. The concentrations for most of the viruses was lowered 3-4 log10 after the treatment. The removal efficiency was even higher up to 5-6 log10 for some viruses, as norovirus GII and astrovirus (Figure 10). This was slightly higher than the results obtained in the study described in Paper III, where one to four log10 reduction was shown for most of the viruses after conventional treatments.

Although a high removal of viruses from the incoming wastewater was achieved at the Rya WWTP, there was still a substantial amount of virus genomes in the treated effluent wastewater. There were up to 2.61×10^5 and 1.76×10^5 virus genome copies per L treated effluent of sapovirus and norovirus GI, respectively (Figure 10). Treated wastewater is usually discharged into receiving water systems, as in Gothenburg into the Göta River. The treated wastewater may also be reused for irrigation in regions where there is an increasing demand of water resources [96]. However, inadequate removal or inactivation of viruses, especially in the irrigation water, may spread the viruses to humans from the irrigated corps, which has been shown in some studies [97, 98]. Human viruses have also been identified in treated wastewater also by others [280, 281]. The infectivity of these viruses is unknown. The detection methods used in this study cannot distinguish between infectious and non-infectious viruses. Further studies are needed to understand if all or part of the viruses in the effluents are infectious.

4.5.4 Virome in effluent wastewater analyzed by NGS

To understand the viral diversity in treated wastewater, the eleven effluents were analyzed by NGS, and compared with the results from qPCR. Between 204,816 and 1,395,590 reads per sample was classified as virus reads. The same NGS analysis workflow was used as in Paper IV. The virus reads accounted for 1.27% to 15.76% of all raw reads. The virus concentration in all samples was relative stable, and ranged from 17 to 110 reads per liter. Sequences homologous to viruses belonging to 31-48 different known virus families were identified in all eleven samples. This was even higher than the number of viruses and virus families identified

in untreated sewage described in Paper III, where 327 viruses from 25 different virus families were found. However, this result does not mean that less viruses existed in the raw sewage, since the NGS methods and data analysis workflow were different between the studies.

A slightly different outcome was observed when comparing the results between qPCR and NGS for 13 common enteric viruses. Nine of the viruses could be detected by both methods in the effluent wastewater, whereas HAV and rotavirus were only detected by qPCR. HEV sequences, on the other hand, were only detected by NGS. PCR or qPCR target specific genomic regions of the viral genomes and is widely used for virus detection since long. However, these nucleic acids based methods face some challenges in detecting viruses from water environments. In such samples there may be a large amount of free DNA as well as humic acids, that can hamper the sensitivity or inhibit the reaction. Also highly dynamic and diversified virus genomes may affect the sensitivity and specificity. NGS uses parallel sequencing approaches and produces high throughput sequences data. Recently it has been used for virus identification and discovery in water environments [130, 132, 246]. The detection of HEV by NGS in this study showed that this technique could overcome the disadvantages of qPCR to some extent. Although NGS substantially improves the possibility to identify viruses, it may still underestimate the virus diversity in water, as was the cases for rotavirus and HAV in this study. Similar results were found in other studies exploring the virome in raw sewage [130, 132].

The most abundant sequences in the treated wastewater were homologous to bacteriophages belonging to the virus families *Microviridae*, *Siphoviridae*, *Myoviridae*, and *Podoviridae*. These sequences accounted for about 43-72% of all virus reads. This was similar to the results obtained in incoming sewage and treated drinking water described in Papers III and IV. There were also many reads homologous to viruses infecting plants. Several belonged to the *Virgaviridae* family, and accounted for up to 37% of all vial reads in one sample (Effluent 1 from March 2017). Interestingly, the longest of contig obtained in this study was a gokushovirus sequence (1,619 bp; *Microviridae*), followed by PMMoV (1,493 bp; *Virgaviridae*). These two viruses were shown to be more widely distributed in different types of water in Sweden than the commonly used surrogate viruses, MS2 and Qβ phages, which were not detected in this thesis (Paper III, IV and V). Gokushovirus and/or PMMoV may therefore be used as viral indicators in the evaluation of water quality and water treatment in Sweden.

4.5.5 Validation of NGS results by detection of HEV and gokushovirus

Two semi-nested PCRs were developed to amplify gokushovirus and HEV sequences in effluent wastewater. There were two reasons to select these two viruses. First, gokushovirus sequences were abundant in all samples, and HEV was not detected by qPCR but by NGS. The detection of these viruses in effluent wastewater by gel PCR followed by sequencing would validate the NGS results. Second, comparisons of the sequences between those identified in this study and those in raw and tap water from Paper IV would provide an overall understanding of the circulation of gokushovirus and HEV in the aquatic environments in Gothenburg. Gokushovirus could be amplified in eight effluent samples, however, the amplificates

contained multiple sequences. Only two sequences could therefore be assembled. HEV could be amplified in seven effluent samples. Sequences from six samples could be assembled. One sample (Effluent 9) contained two different HEV sequences. In total there were seven HEV strains identified in the treated water.
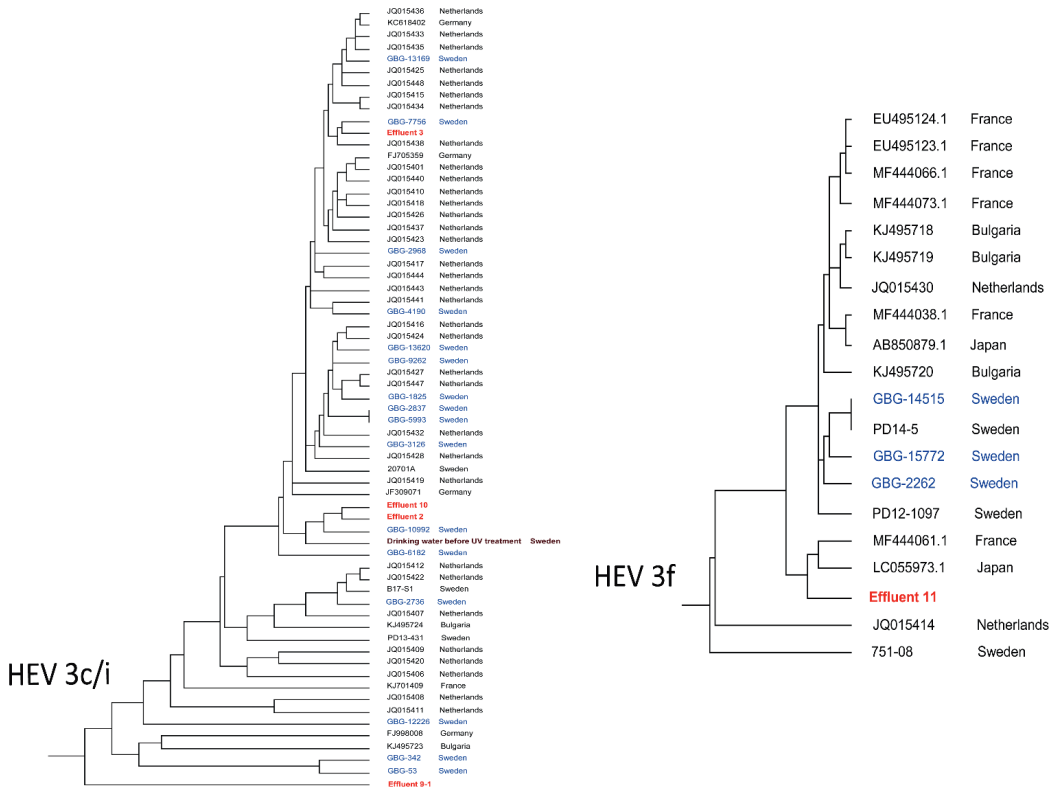

Figure 11. Branch of HEV3 subtype 3c/i and 3f from a phylogenetic analysis based on 311 nucleotides of partial ORF1 in 346 HEV strains. The strains from treated effluent wastewater are shown in red. Strains from Swedish patients are shown in blue. One strain from treated water at a drinking water treatment plant in Gothenburg is shown in dark red.

Phylogenetic analysis for HEV showed that five strains were HEV3 and the other two strains were rat HEV. Four of the HEV3 strains were subtype 3c/i. Three of these (from Effluent 2, 3 and 10) were genetically similar to strains from Swedish patients diagnosed in 2014 and 2015 (Figure 11). This subtype is often isolated from Swedish chronic HEV carriers, and has not been identified in Swedish pigs or wild boars. Two of these HEV3c/i strains (from Effluent 2 and 10) formed a clade with one strain from a Swedish patient and the strain identified in water after carbon filtration from Alelyckan DWTP (Figure 11). This suggests waterborne infection also for HEV3, which has not been reported previously. The fifth HEV3 strain was of subtype 3f (Figure 11), which is commonly isolated from acute human HEV cases and Swedish pigs

and wild boars. The identification of more HEV3c/i strains than the more common subtype HEV3f in both this study and in Paper IV, indicates that HEV3c/i strains may be more easily spread by water than the other HEV3 subtypes. Another reason could be that this subtype is more common than anticipated due to higher number of subclinical infections or excreted in higher amount than the other subtypes. Different HEV3 subtypes may also have different sensitivity to the treatments of the water, with HEV3c/i strains being the least responsive to the treatment.

The two rat HEV strains formed a separate clade between the two clades formed by European and Asian rat HEVs, and may represent a new type of rat HEV. Several wild rat species not only carry rat HEV, but also HEV3. A recent study from Italy detected HEV3 in black rats caught in pig farms. That strain was identical to HEV strains from the swine in the same farm, indicating that wild rats may play a role in zoonotic transmission of HEV3 [226, 282]. In addition, recent works have shown human acute hepatitis in immunocompetent patient, and chronic hepatitis in immunocompromised patient caused by rat HEV [283, 284]. The public health risk of rat HEV is still not well understood. Since there is no study on the prevalence of rat HEV or other zoonotic HEV genotypes in Swedish rodent animals thus far, further investigation is necessary, and the potential of HEV cross-species transmission needs to be evaluated.

CONCLUSIONS

Paper I

We showed that HEV infections are common in wild boars living within the Barcelona metropolitan area in Spain, and in southern Sweden. All infections were caused by HEV3 but with different subtype distribution between countries. Most Spanish strains were of subtype 3c/i, whereas most Swedish strains were of subtype 3f. Some strains from the wild boars were genetically similar to strains found in pigs and patients from several European countries. One strain from Spain was similar to strains from chronically infected humans. This raised concerns that wild boars may pose a higher risk for zoonotic HEV transmission than previously anticipated. With continuous expanding wild boar populations in Europe, direct or indirect contacts between wild boars and humans are increasing. There is thus a need to monitor HEV infection in wild boar populations to investigate if transmission to humans is common.

Paper II

HEV was shown be prevalent in Swedish domestic pigs. In total 77% of the investigated farms had HEV-infected piglets. HEV RNA was detected in 22% of the fecal samples from these piglets. There was no difference in prevalence of HEV between types of farm. Analysis of the strains revealed that each pig farm had a unique HEV3 strain, and that strain remained in the farm and did not change over time. Some of the strains were intermixed with strains from Swedish patients and wild boars in the phylogenetic tree, indicating a zoonotic transmission of HEV. The finding of farm-specific strains could help to identify source of HEV infection down to farm level. The detection of HEV may also be used as a marker for the cleaning routines of the stables.

Paper III

In this study, an in-house concentration technique to concentrate viruses in larger volumes of water was developed. This technique could concentrate HAdV2 between 100 to 1,000 times. The method was used to investigate the efficiency to remove viruses from wastewater in one WWTP in Knivsta. This WWTP used additional ozone treatment after conventional treatments during the study period. We showed that the conventional treatment reduced the amount of viruses with one to four log10, apart from adenovirus and parvovirus, for which the removal was less efficient. Ozone treatment reduced the concentration of viruses with an additional one to two log10, however less for adenovirus. The virome in influent wastewater was explored by NGS, where 327 viruses belonging to 25 families were identified. These viruses could infect human, plant, insect, and bacteria. Bacteriophages accounted for the largest proportion, and the human related viruses identified were mainly associated with enteric disease. Sequencing of the adenoviruses revealed a complex composition in the influent wastewater. Different types of viruses may have varying degrees of sensibility to conventional and ozone-treatments. This study expands our knowledge of the usage of ozone treatment to eliminate viruses from water.

Paper IV

The efficiency to remove viruses at two Swedish drinking water treatment plants in Gothenburg, was evaluated. At Lackarebäck DWTP, combined conventional and UF membranes lowered the amount of viruses with 4.3 log10. There was a 3.2 log10 reduction of viruses at Alelyckan DWTP which combined conventional and UV treatments. Although the number of viruses were significantly reduced at the two DWTPs, there were sequences representing many different virus families, including HEV, in the treated drinking water and in tap water. The risk of HEV infection through drinking tap water is probably negligible since its amount, corresponding to 10-130 International Units of HEV RNA/mL, in tap water was low. However, HEV strains in the water were of subtype HEV3a and HEV3c/i. The latter is associated with an unknown source of infection in humans in Sweden. It has not been detected among its major reservoirs, as domestic pigs and wild boars, indicating water may play a role in HEV3 transmission. This route should be considered for patients with unknown route of infection in developed countries. The analysis of the virome in drinking water (Paper IV) and wastewater (Paper III, V) showed abundant sequences homologous to gokushovirus and pepper mild mottle virus in all types of waters. These viruses could potentially be used as viral indicators of the water quality, at least in Sweden.

Paper V

The presence of 13 common human enteric viruses in incoming and treated wastewater at Rya WWTP, located in Gothenburg, was monitored monthly during 2017. Twelve viruses were detected either by real-time PCR or NGS. There was a general 3-6 log10 reduction of virus concentration with the treatment of the wastewater. Two types of seasonal variations were observed in incoming wastewater. One with a winter seasonal trend, as for norovirus GII and GIV, astrovirus, rotavirus and sapovirus. The other trend was a relative stable amount of viruses throughout the year, as for aichi virus, parechovirus, enterovirus, and norovirus GI. The presence of human enteric viruses in wastewater is a reflection of their circulation in the communities. Some viruses, as parechovirus and aichi virus, were detected in wastewater, but are not routinely diagnosed for at Swedish hospital laboratories. They could be the causes of undiagnosed gastroenteritis infections. We also showed that although there were similar seasonal patterns, there was a large difference in the number of estimated and diagnosed persons infected with the viruses. For norovirus GII, the difference was very large during some periods. This may be due to the presence of many infected persons who do not seek medical care due to mild infections. Altogether this study suggests that routine monitoring of certain viruses in wastewater could be a supplementary tool to understand the viral epidemics in the society. In addition, seven HEV strains belonging to multiple subtypes were detected in effluent wastewater. It was noted that two HEV3 strains were on the same clade with strains isolated from DWTP in same area, and from Swedish patients, indicating waterborne transmission for HEV3, which should be taken into consideration when investigating source of infections.
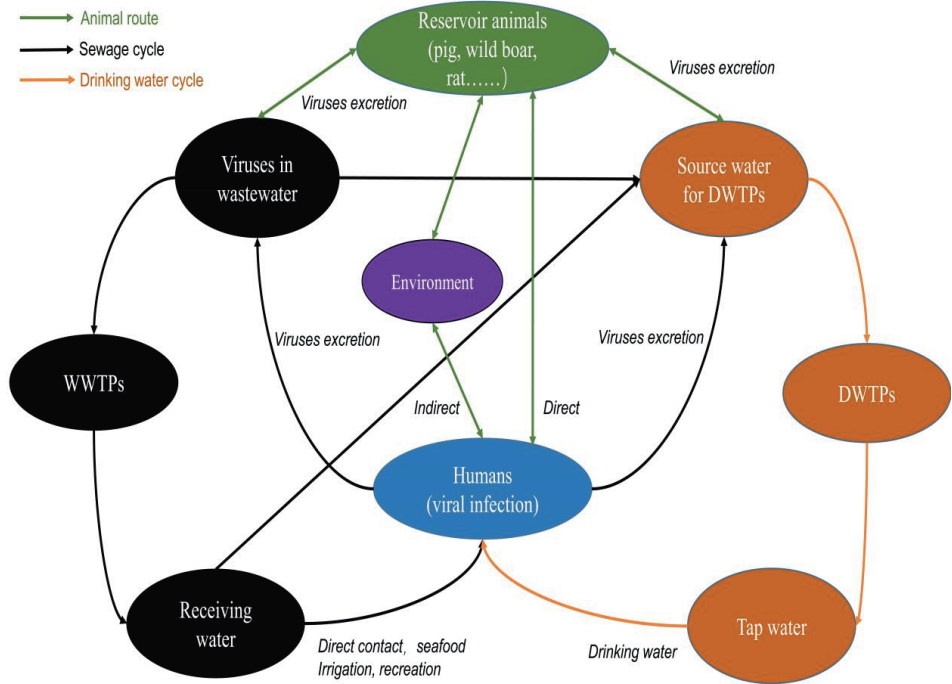
Figure 12. The circulation of human enteric viruses in humans, animals, drinking water, and wastewater.

At the end, I would like to summarize using a straightforward schematic diagram (Figure 12). This figure illustrates the transmission routes of human enteric viruses in humans, animals, drinking water, and wastewater, which were investigated in this thesis. This thesis provides knowledge about how enteric viruses are disseminated in the population through animals, drinking water and wastewater.

FUTURE PERSPECTIVES

This thesis showed that HEV is common in its reservoir animals, as domestic pigs and wild boars, and there is a risk for zoonotic transmission between domestic pigs and wild boars, as well as to humans. Continuous monitoring of HEV in the reservoir animals should be carried out, which could be helpful to control and prevent the risks of HEV spread to society, especially with the expanding territory and population of wild boar in Sweden.

Rat HEV strains were identified in effluent wastewater together with HEV3 strains, and these strains were genetically different from the current European and Asian rat HEV groups, indicating the presence of a new type of rat HEV in Sweden. However, the HEV prevalence in Swedish rodents and what types of HEV they carry is not known, and its role of HEV transmission is not clear. Previous studies showed that the wild rats could simultaneously carry rat HEV and HEV3 strains, and rats with HEV3 were captured around pig farms in several countries [225, 226, 282]. It was suspected that rodent animals may act as an intermediate host of HEV transmission from wild animals, such as wild boar, deer, to domestic pigs, or even to humans. This has been overlooked thus far. The further research on HEV circulation in Swedish rodents may clarify their roles of HEV transmission.

Several common human enteric viruses were detected in effluent wastewater, including HEV that was also found in treated water from DWTPs and in tap water. However, the viability of these viruses after treatment is unknown since all the methods used in this thesis, as qPCR, nested PCR, and NGS, could not distinguish the infectious and non-infectious viruses. The quantification of virus viability in treated water is important to evaluate the risks of infection through drinking tap water, or reusing the reclaimed wastewater. Such analyses should be performed in the future. Future plan is to start viability analyses as cell cultures for some of the human pathogens detected. The integrated cell culture qPCR (ICC-qPCR) has shown the capable to determine the infectivity for some viruses, like adenovirus and astrovirus [285, 286], which will be tested in further studies. Some previous studies have also described the application of ICC-qPCR for HEV. However, the HEV strains they used were derived from the human hepatoma cell line HepG2/C3A, which contains a 171-nucleotide insertion that encodes 58 amino acids of the human S17 ribosomal protein to promote the growth [287]. For wild type HEV strains isolated from environmental samples, especially in tap water, HEV propagation in cell culture is very difficult. Therefore, some other methods, such as 3D cell culture, will be evaluated in the application of assessment the HEV infectivity.

HEV3 was not only detected in treated wastewater, but also in water from DWTPs and tap water from the same area. Subsequent phylogenetic analysis showed that some strains from DWTP and WWTP were genetically close to each other, and to strains isolated from Swedish patients. This raised concerns of also waterborne transmission of HEV3 in developed countries besides the well-known zoonotic transmission. There were multiple subtypes, as HEV3a and HEV3c/i, in different water, with 3c/i as the most prevalent. It is unknown if some subtypes are more easily spread through water than other subtypes, and the lowest human infectious dose of HEV3 is not clear. Thus further studies to answer these questions are needed to

understand and control the potential risks of HEV3 transmission through water.

A diversity of human enteric viruses was detected in incoming wastewater and treated wastewater, their presences in wastewater is a reflection of their circulation in the communities. Some viruses, as parechovirus and aichi virus, were detected in wastewater, indicating a circulation of these viruses in the population, but they are currently not routinely tested for at local hospital laboratories. This means that their circulations is ignored and they could cause undiagnosed gastroenteritis infections. Further investigations on their prevalence in the general Swedish population and in patients with gastroenteritis would illustrate their roles in public health significance. In addition, monitoring of enteric viruses in wastewater should be continued to be used as a supplementary tool for an early warning of upcoming virus outbreaks in the society.

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