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Public health risks associated with hepatitis E virus (HEV) as a food-borne pathogen

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Abstract

Hepatitis E virus (HEV) is an important infection in humans in EU/EEA countries, and over the last 10 years more than 21,000 acute clinical cases with 28 fatalities have been notified with an overall 10-fold increase in reported HEV cases; the majority (80%) of cases were reported from France, Germany and the UK. However, as infection in humans is not notifiable in all Member States, and surveillance differs between countries, the number of reported cases is not comparable and the true number of cases would probably be higher. Food-borne transmission of HEV appears to be a major route in Europe; pigs and wild boars are the main source of HEV. Outbreaks and sporadic cases have been identified in immune-competent persons as well as in recognised risk groups such as those with pre-existing liver damage, immunosuppressive illness or receiving immunosuppressive treatments. The opinion reviews current methods for the detection, identification, characterisation and tracing of HEV in food-producing animals and foods, reviews literature on HEV reservoirs and food-borne pathways, examines information on the epidemiology of HEV and its occurrence and persistence in foods, and investigates possible control measures along the food chain. Presently, the only efficient control option for HEV infection from consumption of meat, liver and products derived from animal reservoirs is sufficient heat treatment. The development of validated quantitative and qualitative detection methods, including infectivity assays and consensus molecular typing protocols, is required for the development of quantitative microbial risk assessments and efficient control measures. More research on the epidemiology and control of HEV in pig herds is required in order to minimise the proportion of pigs that remain viraemic or carry high levels of virus in intestinal contents at the time of slaughter. Consumption of raw pig, wild boar and deer meat products should be avoided.

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Summary

The Panel on Biological Hazards initiated a self-tasking mandate following the requirement of the European Food Safety Authority (EFSA) in order to provide information on the occurrence and control of hepatitis E virus (HEV) as a food-borne pathogen. The opinion is a critical evaluation of available information on HEV, the methodologies for the detection, characterisation and quantification, the prevalence of HEV in relevant food animals and the importance of food as a source of infection. The opinion draws conclusions on the four terms of reference requested: (1) to critically review current methods for the detection, identification, characterisation and tracing of HEV in food-producing animals and foods; (2) to identify HEV reservoirs focusing on genotypes of public health significance to determine the importance of food-borne pathways and identify potential control options; (3) review the epidemiology, occurrence and persistence in foods and consumer habits contributing to infection; and finally, (4) to investigate possible control measures along the food chain and evaluate decontamination treatments.

A literature search and critical review process was used to gather scientific publications, reports and official documents relevant for this opinion. The qualitative evaluations was augmented by the knowledge and expertise of the Working Group (WG) members and information about relevant surveillance data provided by members of the EFSA Biological Hazards (BIOHAZ) Panel and the European Centre for Disease Prevention and control (ECDC).

Food-borne transmission of HEV appears to be a major route in Europe. Hepatitis E (HE) is an important infection in humans in the European Union (EU)/European Economic Area (EEA) countries, reported to cause more than 21,000 (mostly locally acquired) clinical cases with 28 fatalities over the last 10 years. An overall 10-fold increase in reported HEV cases in EU/EEA countries has been observed; the majority (80%) of cases were reported from France, Germany and the UK. However, as infection in humans is not notifiable in all Member States, and surveillance differs between countries, the number of reported cases is not comparable and the true number of cases would probably be higher. Twenty countries already have surveillance systems in place to record the number of acute, chronic and fatal cases as well as case-based clinical data to better understand the underlying epidemiology and trends of the disease. Immunosuppressed patients are at increased risk of developing chronic infection with severe disease progression and fatal outcomes. HE viral infections resulting in severe, fulminant hepatitis, and recently extrahepatic manifestations, particularly neurological and haematological disorders, have been described in patients with both acute and chronic HEV-3 infection.

The species *Orthohepevirus A* includes two genotypes of HEV originating from humans only (HEV-1 and HEV-2), two genotypes reported from both humans and different animal species (HEV-3 and HEV-4). These latter are associated with food-borne infections linked to pigs, wild boar and deer meat. Other recently described closely related strains with more limited public health relevance have been found in a range of animals including, rabbits and camels.

In EU/EEA, genotype 3 viruses with the most common subtypes being HEV-3c and HEV-3e, f, g, and very few cases of HEV-4, have been described in humans and in domestic pigs which appears to represent the main source of zoonotic transmission of HEV in Europe. Molecular evidence of the source of transmission and relationship of viruses is provided by studies that compared virus sequences and subtypes derived from human cases with viruses from pigs, wild boar and deer or consumed products thereof. Some rabbit HEV strains appear to have close sequence homology with some strains found in people and can be experimentally transferred to pigs and primates but the importance of natural transfer of HEV from rabbits to people has not been conclusively determined.

A diversity of methods for HEV extraction, RNA purification and viral genome quantification from a range of food and water samples is available; however, standardisation and systematic method comparison and interlaboratory validation have not been performed for most virus extraction methods. Pigs and wild boars are the main source of HEV in Europe, consequently, the validation and standardisation of methods for virus from pig meat and meat products should be a high priority. Also, extraction methods for other food matrices (shellfish, vegetables and fruit, food surfaces) and bottled water as described in ISO15216 should be validated in order to demonstrate their suitability for the detection of HEV. A need for harmonised typing, subtyping, strain comparison and source attribution methods has been identified. The recent definition of HEV subtype reference strains, a set of whole genome reference sequences for HEV-1 to HEV-7 subtypes and the current development of a web-based typing tool HEVnet represents important steps towards harmonisation. Strains originating from animals, food and the environment should be included in order to enable source attribution studies.

Thresholds for definition of types, subtypes and 'identical' strains based on sequence comparisons need to be clearly defined to facilitate the investigation of HEV transmission chains.

Several tests available for detection of immunoglobulin (Ig)M or IgG in human and animal sera have been established. However, as test characteristics vary between the different serological assays, it is important to consider these when the results are interpreted.

The development of efficient cell culture methods for HEV should be encouraged to facilitate acquisition of quantitative data on infectivity, inactivation and survival of HEV in food and in the environment. Reports on successful HEV isolation from food samples in cell culture are rare, and no standardised or validated method for the preparation of food samples before inoculation into cell cultures is available.

Prevalence of HEV varies greatly between pig farms, production systems and countries, with small non-industrialised production being at higher risk in some studies. In general, however, the HEV between-herd, as well as within-herd prevalence, is often very high. A proportion of pigs, likely to be less than 10%, remain viraemic at slaughter, which is a probable cause of prime meat cuts containing HEV. Occupationally exposed humans having contact with the animal reservoirs show higher seroprevalence than the general population, providing evidence for zoonotic transmission from animals to humans.

Food-borne transmissions of HEV linked to consumption of uncooked or undercooked pork and wild boar meat, liver and liver sausages have been definitively proved. The number of descriptions of HEV infections in humans linked to meat or liver consumption remains limited, and further studies are needed to identify if HEV occurs also in other foods of animal or non-animal origin. The persistence of HEV in liver and processed meat products, not subjected to cooking but to process technologies (cured, smoked, dried, fermented) is unknown. Also, the role of the environment (e.g. organic fertilisers or irrigation water) as source for HEV contamination of food of non-animal origin is unknown and should be evaluated.

Presently, the only efficient control option for HEV infection from consumption of meat, liver and products derived from animal reservoirs is sufficient heat treatment. Results obtained to date suggest that heat resistance (kinetic of reduction of infectivity) of HEV is variable, depending on the strain or genotype and the matrix studied (e.g. meat, sausages, by-products, mussels). In addition, viral HE particles can exist as non-enveloped or 'quasi-enveloped' virions, and the envelope may also influence the stability of the viral particle in meat, liver or blood food products. Different combinations of temperature and time have been investigated in the inactivation of HEV-3, dependent on the used matrix. Conditions for heat treatment should be validated for the specific ability to inactivate HEV under commercial conditions.

HEV is sensitive to current water disinfection treatments using chlorination and UV irradiation, similar to other viruses. These measures can be also used to minimise cross-contamination through treatment of food contact surfaces, decontamination of water for irrigation or shellfish mussel depuration.

There are several studies that identify regional consumption habits such as consumption of raw or undercooked pork or wild boar products (e.g. sausages, salami), as risk factors for HEV infection in Europe. There are indications that the human population of Central European countries have a higher seroprevalence than, for example, Nordic populations.

Regulations related to hygienic measures for foods of animal origin and control of products of animal origin for consumption are laid down in EU Regulations 853/2004 and 854/2004. Ante-mortem and post-mortem inspections are not able to detect HEV infection, which can be present in liver or meat at the time of slaughter. Some management-based methods such as batching of farrowing and weaned pigs to influence the time of first exposure to HEV and farm decontamination measures for potential reduction of HEV risk in pig herds have been described, but need to be confirmed in well-designed intervention studies.

A recombinant subunit vaccine based on a HEV-1 peptide strain was registered for humans in China in 2011, yet has not been licensed or approved in other countries. Vaccination of pigs is a potential control option, but the effect of vaccination of pigs as a possible method to prevent human infection requires further investigation. No vaccine for animals is commercially available currently.

Despite considerable research in recent years, the level of awareness of HEV risk associated with pig meat products and other reservoirs and sources is low, so dissemination of information and advice to consumers and those working with potential sources of infection should be optimised. In particular, provision of information on the risk of consumption of raw or undercooked pig, wild boar and deer products to vulnerable groups (e.g. persons with a weakened immune system or pre-existing liver

damage) may help prevent the most serious HEV infections. In order to minimise the risk of an HEV infection, consumers should thoroughly cook meat and offal, especially pork, wild boar and deer meat products.

The development of validated quantitative and qualitative detection methods, including infectivity assays, and consensus molecular typing protocols are required for the development of quantitative microbial risk assessments and efficient control measures.

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1. Introduction

1.1. Background and Terms of Reference as provided by European Food Safety Authority (EFSA)

Hepatitis E virus (HEV) is the most common cause of enteric viral hepatitis infection worldwide (Lapa et al., 2015). In most cases, HEV infections are asymptomatic; the virus may, however, be responsible for mild to fulminant acute hepatitis (fatality rates generally under 0.5% but may reach up to 25% in pregnant women) and also chronic hepatitis in immunocompromised patients; also an association to neurological symptoms and syndromes has been suggested. Even after more than 15 years of studies describing clinical cases of autochthonous HEV in Europe (i.e. European Economic Area (EEA) countries), HEV infection is an underdiagnosed disease and, in part due to the lack of a routine protocol for the analysis and diagnosis for such infections in many European clinical centres, with a corresponding underestimation of the incidence of clinical cases of HEV infections (Kamar et al., 2014). The environmental contamination from human/animal sources may have a role in the dissemination of HEV, and the virus has been detected in urban sewage and, with high frequency, in sewage and sludge from pig slaughterhouses in Europe (Clemente-Casares et al., 2003; Rutjes et al., 2009).

Data regarding the burden of HEV infections are limited owing to the lack of HEV surveillance in many European countries (i.e. EEA countries). The anti-HEV antibody seroprevalence varies greatly, depending on the geographic area considered and the populations studied, as well as the assay used in anti-HEV immunoglobulin G (IgG) detection. Anti-HEV IgG prevalence in blood donors ranged from 1.3% (Italy) to 22.4% (France). Contact with pigs or consumption of undercooked or raw pork meat are recognised risk factors for transmission of infection, given the high seroprevalence observed in pig veterinarians, pig farmers, and in the populations that usually consume uncooked pork (reviewed in Lapa et al., 2015). There is an urgent need for a realistic evaluation of the burden of HEV disease in humans in general and in specific risk groups (e.g. immunocompromised individuals).

There are four known genotypes of HEV infecting humans, designated HEV-1 to HEV-4. Genotypes 1 and 2 are isolated in human epidemic outbreaks in low income countries, while genotypes 3 and 4 have been isolated in humans and in wild and farmed food animals, in both low- and high-income countries. Autochthonous infections in Europe (EEA) with genotype 3 (and 4 with lower prevalence) are increasingly being recognised as the cause of unexplained liver disease in many countries (Arends et al., 2014). Autochthonous HEV infection is considered an emerging disease in Europe, and the circulation of the virus (genotypes 3 and 4) in human and animals (swine, wild boar and deer) has been confirmed. Severe cases of fulminant autochthonous HEV infection caused by genotype 3 have been reported, often occurring in immune-compromised subjects or in those with chronic liver disease (Lapa et al., 2015).

Hepatitis E viruses are mainly transmitted via faecal–oral and food-borne routes. This transmission could result from either the ingestion of raw or undercooked meat of infected animals, ingestion of fruit or vegetables washed or irrigated with contaminated water, or bivalves (mussels, cockles and oysters) collected or maintained in contaminated water. Several studies have demonstrated the food-borne transmission of HEV genotypes 3 and 4 through ingestion of meat from deer, wild boar and pig meat (Japan, Spain, Italy), ingestion of pig liver sausage (France) and also contaminated shellfish (UK passengers in a world cruise, Japan). HEV in pig liver sold in retail locations has been reported from Japan, USA, Korea, India, the Netherlands and England. HEV prevalence in pig production regions in Europe, as well as within herds of domestic swine, is often very high (98%, 204/208 positive herds in Spain; 55% of 97 herds positive in the Netherlands; Pavio et al., 2010), and HEV genotype 3 has been detected in domestic swine in many high-income countries. Domestic swine appears to represent the main source of direct zoonotic transmission of HEV (Pavio et al., 2010; EFSA BIOHAZ Panel, 2011a; Van der Poel, 2014).

There is a need to review the available methodologies for the detection, characterisation and quantification of HEV and the prevalence of HEV in relevant farm stock, for example, pigs and importance of food as a source of infection. Such data are necessary for risk assessment activities that will assist risk managers in making informed decisions on the most appropriate risk mitigation measures needed to control HEV transmission from food animals and food to humans.

In 2011, EFSA published an 'Opinion on the occurrence and control of food-borne viruses, including HEV' (EFSA BIOHAZ Panel, 2011a). New information has subsequently become available including data

on occurrence, stability and methods that can be taken into account for an update on the risk of HEV infection to public health.

Terms of Reference

The Panel on Biological Hazards (BIOHAZ Panel) is requested to issue a Scientific Opinion on public health risk associated with HEV as a food-borne pathogen. In particular, the BIOHAZ Panel is requested:

- 1) To critically review current methods for the detection, identification, characterisation and tracing of HEV. In the case of methods for detection, special emphasis should be made to assess their application to samples from food-producing animals and foods.
- 2) To review the scientific literature on HEV reservoirs (human, animal and environmental) focusing on genotypes of public health significance in order to determine the importance of food-borne pathways and identify potential control options.
- 3) To examine currently available information on the geographical distribution of HEV and epidemiology, occurrence and persistence in foods and consumer habits contributing to infection.
- 4) To investigate possible control measures along the food chain and evaluate decontamination treatments.

1.2. Approach to answering the Terms of Reference

The terms of reference have been interpreted to describe and analyse the new available information on detection and quantification methods of HEV in food currently in use in research laboratories, including cell culture methods for infectivity assays; information on new strains and animal reservoirs recently described; the available data on the prevalence and levels of HEV as food contaminant; and the food products known to be associated to HEV infections and outbreaks in humans have been reviewed. The information available on the environmental sources of infection, in addition to food and food products, has been updated. Finally, disinfection treatments and potential control measures are described and discussed to produce recommendations for reducing HEV-associated risk in food products and consumers.

Term of reference 2 'review the scientific literature on HEV reservoirs (human, animal and environmental)' will be answered by analysing the available information on the animal and human reservoirs of the HEV. The concept 'reservoir' is used referring to the hosts where the viral infection is maintained in the long term. In addition, environmental sources of infection are also frequently referred to as environmental reservoirs using in this case the term 'reservoir' for the places where large amounts of a pathogen may be present. Environmental sources of HEV infections as HEV-contaminated environments including animal manure, human sewage and faecal-contaminated water will also be considered in this report.

2. Data and methodologies

A non-systematic literature review was used to gather scientific publications, reports and official documents relevant for this opinion. In general, the qualitative evaluation by means of literature review was carried out based on the knowledge and expertise of the Working Group (WG) members. The experts in the WG selected relevant references starting from review papers, book chapters and peer-reviewed papers retrieved through non-systematic searches, and increasing the number of papers through 'footnote chasing' (White et al., 1992) until reaching a coverage of the subject considered sufficient by the WG. Considerable use was made of ongoing citation input by WG members and information about relevant publications provided by members of the EFSA BIOHAZ Panel.

3. Assessment

3.1. Characteristics of HEV

Hepatitis E virus, the pathogen causing acute hepatitis E (HE), has become a worldwide public health concern. Globally, HEV is the most common cause of acute viral hepatitis. HEV infection, mainly genotype 3, is increasingly reported in Europe where it affects mostly older male adults (Lewis et al., 2010; Adlhoch et al., 2016). Transmission can occur via contaminated drinking water (HEV-1 and HEV-2)

or contaminated food (HEV-3 and HEV-4; Hakim et al., 2017). Human-to-human transmission has been sporadically observed, and can be transfusion or transplantation-related. The majority of the infections are asymptomatic but are accompanied by seroconversion. In acute cases, the disease is a self-limiting hepatitis. However, in rare cases, acute HE can result in a severe, fulminant hepatitis (acute liver failure; Festa et al., 2014). Cases of chronic HE infection due to genotype 3 or 4 have been reported in immunosuppressed people, particularly organ transplant recipients on immunosuppressive drugs (Netzler et al., 2016). Recently, extrahepatic manifestations, particularly neurological and haematological disorders, have been described in patients with acute and chronic HEV-3 infection (Kamar et al., 2012; Woolson et al., 2014; Khuroo et al., 2016; WHO, 2016).

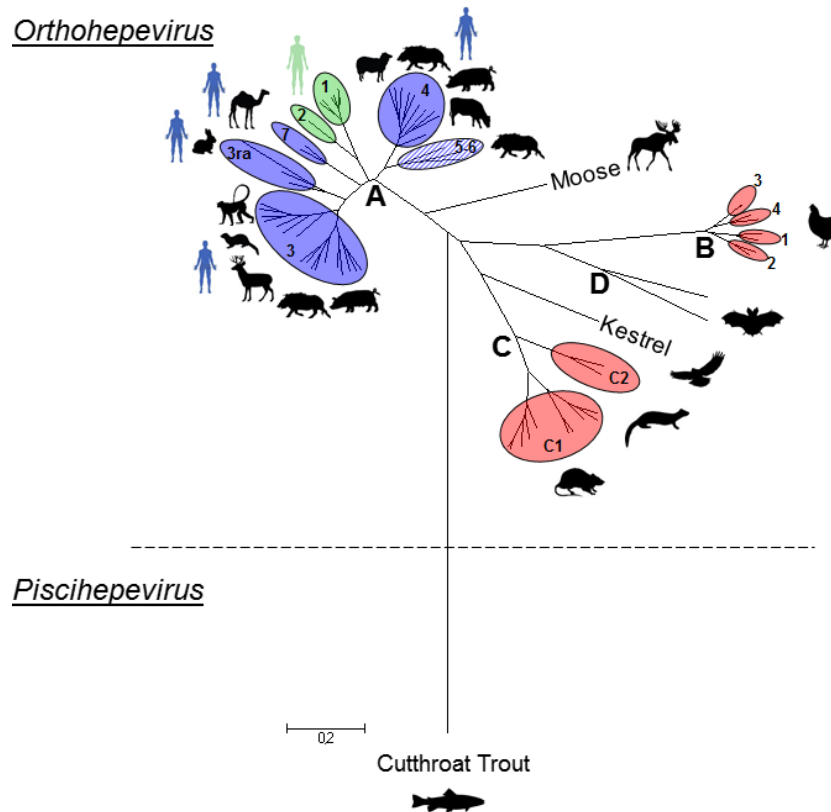
HEV belongs to the Hepeviridae family; positive-stranded RNA viruses that infect a wide range of mammalian species, as well as chickens and trout. Hepeviridae includes two genera: *Orthohepevirus* (mammalian and avian species) and *Piscihepevirus* (infecting trout). Most of the HEV strains identified so far belong to the *Orthohepevirus* genus that is divided into four species A, B, C and D (Smith et al., 2014), with new sequences identified in moose and kestrel not yet assigned to species (Figure 1; Doceul et al., 2016). The viruses of the species *Orthohepevirus* A include HEV found in humans, pigs, wild boar, deer, mongoose and camels. The other species in the genera are *Orthohepevirus* B (including the virus from chicken) *Orthohepevirus* C (HEV found in rat, greater bandicoot, Asian musk shrew, ferret and mink) and *Orthohepevirus* D (comprising HEV from bats) (Smith et al., 2014). More HEV-related sequences are expected to be described in the future, for example, the results of a study of viruses in sewage using metagenomics showed an incomplete virus genome sequence that suggests that an additional genus in the family Hepeviridae may exist, although further confirmation of these results and the identification of the host are required (Ng et al., 2012).

The HEV virion is a small, spherical particle of approximately 27–34 nm in diameter and has a single-stranded, positive sense ribonucleic acid (RNA) genome surrounded by an icosahedral capsid (Reyes et al., 1990; Kamar et al., 2012). HEV was initially isolated from a patient's stool and visualised by immunoelectron microscopy as non-enveloped particles. However, recent studies have shown that viral particles circulating in the bloodstream and in culture medium are cloaked in a host cell membrane (Takahashi et al., 2010). These novel virus particles are infectious, yet they do not carry viral antigens on the surface and are completely resistant to neutralising antibodies in standard neutralisation assays. This dual lifestyle is similar to that of hepatitis A virus (HAV), another hepatotropic virus that is phylogenetically unrelated to HEV. The enveloped HEV particles are termed 'quasi-enveloped' virions or eHEV, and the envelope probably reduces its attachment to permissive cells, thereby reducing the risk of infection and influencing the stability of the viral particle (Takahashi et al., 2010; Qi et al., 2015; Yin et al., 2016).

The viral genomes are 6.6–7.3 kb long, consisting of a short 5' untranslated region (27–35 nt), three discontinuous and partially overlapping open reading frames (ORFs) 1, 2 and 3, and a short 3' untranslated region (65–74 nt) that is terminated by a polyadenylated tract. The longest ORF (ORF1) encodes for viral non-structural proteins carrying domains with methyl transferase, helicase and replicase activities. ORF1 is followed by ORF2, which encodes the capsid protein of about 660 amino acids, and ORF3, which overlaps with ORF2 and encodes a phosphoprotein of about 114 amino acids that modulates cellular activities and is putatively responsible for the virion's egress from infected cells (Ahmad et al., 2011; Okamoto, 2011).

3.1.1. HEV phylogeny and genetic diversity

Orthohepevirus A includes two genotypes of HEV originating from humans only (HEV-1 and HEV-2), two genotypes reported from both human and different animal species, associated with zoonotic cases (HEV-3 and HEV-4), two genotypes from wild boar in Japan (genotypes HEV-5 and HEV-6), and a single HEV from a dromedary camel (genotype HEV-7) identified in the camel and in a linked human clinical case in the Middle East (Lee et al., 2016). Rabbit HEV and closely related human virus have been placed as distant members within HEV-3 (Zhao et al., 2009; Khuroo et al., 2016). Very recently, a putative new HEV genotype (HEV-8) has been identified in 3 of 305 faecal samples of Bactrian camels from China (Woo et al., 2016). A global view of the unrooted tree of the HEV clades is shown in Figure 1.



The name of different *Orthohepevirus* species (A–D) is added at the junction of the last common ancestor for each species. Genotypes of non-zoonotic HEV species (red), genotypes including HEV strains originating from animals and humans (blue), genotypes infecting humans only (green), and genotypes infecting wild boar that are not linked to human infections (striped blue). Copyright ©2016 by the authors; licensee MDPI, Basel, Switzerland (Doceul et al., 2016).

Figure 1: Phylogenetic tree of representative members of the Hepeviridae family, which is divided into two genera: *Orthohepevirus* and *Piscihepevirus*

Tracing the evolutionary ancestry of HEV has proved difficult; since its identification in 1992, it has been reclassified several times, and confusion still remains surrounding its origins and ancestry. However, a recent study has identified indications of an ancient recombination event and new viral families would have been created by recombination at the junction of the genome that encodes structural and non-structural proteins, and such recombination events are involved in the genesis of HEV, astrovirus and rubella virus (Netzler et al., 2016). The separation in anthroptropic (HEV-1 and HEV-2) and enzoonotic (HEV-3 and HEV-4) forms may have occurred more than 500 years ago (Purdy and Khudyakov, 2010).

Smith et al. (2014) have presented a consensus taxonomic framework that provides the basis for the classification of currently described HEV variants considering phylogenetic relationships, sequence identity and host range, and propose the use of a common reference sequence and numbering system. Phylogenetically, HEV has been classified into four major genotypes. Geographically, HEV-1 was found in tropical and several subtropical countries in Asia and Africa, and HEV-2 originated from Mexico, Nigeria, and Chad; whereas HEV-3 was identified almost worldwide, including Asia, Europe, Oceania, North and South America. In contrast, HEV-4 was found almost exclusively in Asia.

It is speculated that HEV-3 originated in the western hemisphere and was imported to several Asian countries such as Japan, Korea and Taiwan, while HEV-4 has been indigenous and likely restricted to Asia. HEV-3 and HEV-4 were not only identified in swine, but also in wildlife such as wild boar and deer. Furthermore, in most areas where genotypes 3 and 4 were characterised, sequences from both humans and animals were highly conserved, indicating they originated from the same sources. At present, both animal reservoirs and humans are hosts for strains that may be considered both human and porcine, wild boar or deer strains.

Several studies have attempted to define subtypes within each genotype (Lu et al., 2006; Jin et al., 2013). Typing of HEV is almost exclusively based on molecular methods, which define genotypes and subtypes (Li et al., 2015b, 2016). Antigenic comparison of these genotypes indicated that HEV-1 to HEV-7 have the same antigenicity and thus represent a single serotype (Li et al., 2016). A putative new HEV genotype (HEV-8) has also been identified in Bactrian camels from China (Woo et al., 2016).

The subtyping scheme proposed by Lu et al. (2006) with designated alphabetised subtypes has been commonly used in epidemiological studies, although limitations have been observed, and more additional labelling systems considering the high number of new strains described have been suggested (Oliveira-Filho et al., 2013; Smith et al., 2013).

More recent analysis of the complete genome sequences available highlights the difficulty in defining discrete boundaries to distinguish all subtypes with consistency (Bouquet et al., 2012; Oliveira-Filho et al., 2013).

Recombination in HEV genomes may also occur in animals or human patients, and chronic HEV infection in immune-compromised individuals has led to the occurrence of viral strains carrying insertions from human genes (Zhang et al., 2016).

With the objective to facilitate comparison between different studies describing subtypes of HEV and facilitating communication between researchers, Smith and co-workers (Smith et al., 2016) recently published a table with proposed complete genome reference sequences for each subtype considering also previous classifications (Lu et al., 2006). At present, there is enough information available (Lu et al., 2006; Vina-Rodriguez et al., 2015; Doceul et al., 2016; Smith et al., 2016) to describe the distribution of the HEV genotypes in different subtypes or clade/groups. Six subgenotypes are described for genotype 1 (1a–1f); two for genotype 2 (2a and 2b); genotype 3 has 20 reference strains for subtyping with a major clade with six subtypes (3a, 3b, 3c, 3h, 3i and 3j) and another clade with the three subtypes (3e–3g). In addition, there are several sequences not clearly assigned to any subtype, including highly divergent sequences such as the rabbit-derived strains in the 3ra clade. HEV genotype 4 is described having 10 reference strains and nine subtypes 4a–4i, and a sequence type without an assigned subtype. The criteria for subtype assignment vary between different genotypes and methodologies, and the pragmatic approach described by Smith and collaborators (Smith et al., 2016) is currently the most feasible approach for subtyping. Updates to this distribution with reference strains will be posted on the International Committee on Taxonomy of Viruses website.¹ Furthermore, a classification based on distribution of HEV-3 in clades I and II and subclades I-A to I-C (Mirazo et al., 2016) has been described, and the available information both for the subtypes and clades in HEV-3 has been comprehensively summarised by Doceul et al. (2016).

Concluding remarks

- The species *Orthohepevirus A* includes two genotypes of HEV originating from humans only (HEV-1 and HEV-2) and two genotypes reported from both humans and different animal species (HEV-3 and HEV-4). The latter are associated with food-borne infections linked to pigs, wild boar and deer meat. Other recently described closely related strains with more limited public health relevance have been found in a range of animals including wild boars (HEV-5 and HEV-6), rabbits (HEV-3ra) and camels (HEV-7 and HEV-8).
- Viral HE particles can exist as non-enveloped or 'quasi-enveloped' virions, and the envelope may also influence the stability of the viral particle.

3.2. Methods of detection, quantification and typing of HEV in food and food-producing animals

Detection methods for HEV in food are based on molecular methods detecting the viral genome or infectivity assays using cell culture or animal inoculation. The main difference between these methods is that the molecular methods detect the genome but give no information if the virus is infectious. For food-producing animals, serological methods for detection of HEV-specific antibodies can be additionally used. Serological methods detect exposure to the virus, including recent infection in the case of IgM. All of these assays can potentially be performed in a qualitative or a quantitative fashion.

Numerous detection and typing methods have been described. However, the degree of method characterisation and validation done for different methods varies largely. No standardised method for detection, quantification or typing of HEV in food is available so far. However, the Subcommittee

¹ <http://talk.ictvonline.org>

'Microbiology' of the International Organisation for Standardisation (ISO/TC34/SC9) launched an enquiry in April 2015 that resulted in a vote for the development of a standardised ISO method for HEV detection in food samples in the future. This method could then be used as the gold standard against which test characteristics for other methods can be estimated.

Measured prevalence is dependent on the characteristics of test methods used, which can be quite variable (Gerber et al., 2014; Jones and Muehlhauser, 2014; De Schryver et al., 2015; Holm et al., 2015; Hartl et al., 2016). Furthermore, different tests aim to detect different things, for example, serological evidence of prior infection, evidence of the presence of viral DNA or evidence, via infectivity assays, of live infectious virus.

The impact of low specificity due to cross-reactivity during antibody detection or RNA contamination of laboratories conducting polymerase chain reaction (PCR)-based detection methods or sensitivity relating to the variable target sequences used as PCR targets is also difficult to assess.

3.2.1. Molecular methods

The molecular methods usually consist of different modules for virus extraction from the specific sample and RNA extraction followed by detection of the HEV genome by nucleic acid amplification techniques (NAT). Typing of HEV strains is usually done by sequencing of reverse transcription (RT)-PCR products and comparison to reference strains. Next-generation sequencing (NGS)-based methods have been developed recently, which have been used for metagenomic identification of HEV in a sample as well as for typing with the aim to use whole genomic sequences as a basis for genotyping.

3.2.1.1. Methods for virus and RNA extraction from food and water

A broad range of different methods has been described for HEV extraction from meat and meat products. For industrially produced pig liver products, this includes mechanical disruption in lysis buffer, centrifugation and magnetic immunobead-based RNA extraction (Bouwknegt et al., 2007), manual homogenisation and column-based RNA extraction (Wenzel et al., 2011), or disruption in lysis buffer using zirconia beads, centrifugation and column-based RNA extraction (Di Bartolo et al., 2012). For meat products like liver sausages, protocols using manual elimination of fat, disruption in phosphate-buffered saline (PBS), centrifugation and column-based RNA extraction (Colson et al., 2010), disruption using stomacher, centrifugation, polyethylene glycol (PEG) precipitation, chloroform-butanol treatment and bead-based RNA extraction (Martin-Latil et al., 2014), or disruption in TRI[®] Reagent using stomacher, chloroform-butanol treatment and bead-based RNA extraction (Szabo et al., 2015) have been described. Systematic comparisons of the different methods by independent laboratories have not been published, although limited comparative studies on the efficiency of selected methods are available (Martinez-Martinez et al., 2011; Martin-Latil et al., 2014; Szabo et al., 2015). Reported detection limits of the methods are 2.9×10^3 HEV genome copies per 5 g raw sausage (Szabo et al., 2015), 5.3×10^4 HEV genome copies per 2 g liver sausage (Szabo et al., 2015) and 8.7×10^3 to 8.7×10^4 HEV genome copies per 3 g figatelli or liver sausage (Martinelli et al., 2015).

The method described by Szabo et al. (2015) has been validated in an interlaboratory ring trial in Germany using artificially HEV-contaminated liver sausages (BVL, 2016). A major drawback of the method validations using artificially contaminated meat or sausage samples is the fact that the virus is added to the external surfaces of the sample. However, as a consequence of animal infection, HEV may be present within the cells of the investigated sample, which is in contrast to most other virus/food combinations that result from external contamination. Therefore, effective lysis of the cellular content of samples is essential in order to efficiently release the virus from the cells before the following detection steps. The quantitative detection of released pig DNA has been suggested as a control parameter for cell lysis (Szabo et al., 2015). In addition, other control reactions have been suggested for standardised virus detection in food samples (D'Agostino et al., 2011; ISO15216-1² and ISO15216-2³).

² ISO/TS 15216-1:2017 Microbiology of food and animal feed – horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR – Part 1: method for quantification. Geneva: International Organization for Standardization.

³ ISO/TS 15216-2:2013 Microbiology of food and animal feed – horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR – Part 2: method for qualitative detection. Geneva: International Organization for Standardization.

However, validation of the use of those standards for HEV detection in food, especially for quantitative analyses, remains a subject of further studies.

Several protocols have been successfully applied for the detection of HEV in shellfish samples. Most of these have used a homogenate prepared from the digestive glands of shellfish as a starting point. This is in accordance with the results of bioaccumulation studies showing that HEV has a 10–100-fold higher concentration in the digestive tissue compared with gills or mantle tissue (Grodzki et al., 2014). The methods used in the following steps of virus extraction varied, and included PEG precipitation (Donia et al., 2012; Grodzki et al., 2014; Gao et al., 2015, 2016), ultracentrifugation (Donia et al., 2012), ultrafiltration (Gao et al., 2015, 2016) or ethanol precipitation (Namsai et al., 2011). The detection of HEV in shellfish after application of the virus extraction method for shellfish specified in ISO15216 has also been described (Mesquita et al., 2016). RNA was subsequently extracted from the virus concentrates by use of silica-based columns or beads, or by phenol/chloroform-based methods. No systematic comparisons of the efficiencies of the methods for detection of HEV in shellfish are available.

Only a few publications report on detection of HEV in berries or leafy green vegetables. For berries, pectinase treatment of solutions obtained after rinsing of the berries has been applied in a first step in most cases. For virus concentration, this was followed by ultrafiltration (Brassard et al., 2012) or PEG precipitation similar to the ISO15216 protocol for soft fruit (Maunula et al., 2013). HEV was detected in leafy green vegetables using a PEG precipitation method similar to the ISO15216 protocol for salad vegetables (Kokkinos et al., 2012, 2016). Silica-based columns or beads were used for nucleic acid extraction from the virus concentrates. The efficiencies of the applied methods have not been systematically compared so far.

Several publications report the detection of HEV in environmental waters samples as well as irrigation and drinking water. The starting volumes used varied largely between 50 mL (Kitajima et al., 2009) and 300 L (Givens et al., 2016). Different filtration methods, most of them applying multiple filtration steps, have been described. This includes combinations of hollow fibre ultrafiltration (Williamson et al., 2011; Marcheggiani et al., 2015), other ultrafiltration devices (Kitajima et al., 2009; Rutjes et al., 2009; Verma and Arankalle, 2010; Givens et al., 2016) and/or positively (Rutjes et al., 2009; Steyer et al., 2011; Li et al., 2014b) or negatively (Kitajima et al., 2009) charged filter membranes. Glass wool filtrations (Kokkinos et al., 2012; Givens et al., 2016) and flocculation at low pH (Kokkinos et al., 2012) have also been described. Nucleic acids are isolated from the virus concentrates using silica-based columns or beads, or by phenol/chloroform-based extraction methods. Systematic comparisons of different methods for HEV detection in water samples are not available.

One study assessed the performance characteristics of a detection method for analysis of bottled mineral water and tap water using samples artificially contaminated with HEV (Martin-Latil et al., 2012). In this study, 500 mL of water was filtered through a positively charged filter membrane, virus particles were lysed on the filter and the RNA was extracted using silica beads. The limit of detection of this method was 7×10^2 to 3.5×10^3 HEV genome copies per 0.5 L bottled water and 3.5×10^3 HEV genome copies per 0.5 L tap water (Martin-Latil et al., 2012).

3.2.1.2. Nucleic acid amplification techniques

For detection of the HEV genome, several NAT have been developed, which include conventional RT-PCR, real-time RT-PCR (RT-qPCR) or loop-mediated isothermal amplification (LAMP) techniques. Most of the assays have been developed for simultaneous detection of genotypes 1–4. These techniques have been used with RNA extracted from human, animal, food or environmental samples (see above). Many of the assays have been characterised by assessing their analytical sensitivity and specificity. However, interlaboratory ring trials to evaluate the diagnostic sensitivity and specificity are mostly missing, especially for their use with food samples. Since 2011, a quantitative WHO standard for HEV RNA NAT-based assays is available, which can be used as assay control and for assay characterisation and standardisation (Baylis et al., 2011a,b, 2013). In addition, ring trials for HEV RNA detection are offered commercially.

Conventional RT-PCR assays (Schlauder et al., 1999; Huang et al., 2002; Preiss et al., 2006) have been available for many years, and may target several different regions of the HEV genome. Besides their use for HEV RNA detection, they are increasingly applied for genotyping and are therefore described in more detail in the following section. Disadvantages of conventional RT-PCRs include high susceptibility to laboratory contamination and comparatively low sensitivity. Comparisons of conventional assays for HEV detection with real-time assays using human and pig samples consistently indicated higher sensitivity for the real-time assays (Zhao et al., 2007; Son et al., 2014). However, an

advantage of the conventional assays is the generation of longer DNA fragments, which are suitable for sequencing and typing. In addition, some of them allow the detection of a broader range of HEV-related viruses by the use of degenerated primer pairs. For example, a nested broad-spectrum RT-PCR was developed, which can detect human and avian HEV strains, and enabled the identification of a formerly unknown HEV species in rats (Johne et al., 2010).

Several RT-qPCR assays for HEV RNA detection have been described (Orru et al., 2004; Ahn et al., 2006; Enouf et al., 2006; Gyarmati et al., 2007), which are increasingly used for the detection of HEV RNA. A RT-qPCR assay originally developed by Jothikumar et al. (2006) has been reported in a WHO international survey to be used by the majority of laboratories for human HEV detection (Baylis et al., 2013). This assay (Jothikumar et al., 2006) has been also reported to have the highest sensitivity when comparing five different RT-qPCR assays on a set of human clinical samples (Mokhtari et al., 2013), and was shown to broadly detect the HEV genotypes 1–7 (Giron-Callejas et al., 2015). The assay was also optimised by the inclusion of a modified probe for better discrimination between negative samples and those containing only low amounts of RNA (Garson et al., 2012). The general advantage of RT-qPCR assays is high sensitivity, lower risk of laboratory contaminations and faster performance as compared with classical RT-PCR. In addition, quantification can be readily done if appropriate standards are used.

Further developments include multiplex RT-qPCR assays, which enable simultaneous detection of HEV and HAV (Tahk et al., 2011; Qiu et al., 2014), or even the parallel detection of 19 human-pathogenic viruses including HEV-3 strains using a nanofluidic RT-qPCR system (Coudray-Meunier et al., 2016). In addition, multiplex assays were developed that allow the simultaneous detection of HEV and internal amplification controls, thus assessing the amplification efficiency of each reaction (Martin-Latil et al., 2012; Vasickova et al., 2012). Protocols for digital RT-PCR assays, which enable quantification without the need of standards, have recently also been published (Martin-Latil et al., 2016; Nicot et al., 2016).

Reverse transcription-LAMP assays, which allow the molecular detection of HEV without the need for a thermocycler device, have also been described (Zhang et al., 2012; Chen et al., 2014). An RT-LAMP assay was recently successfully used for the analysis of shellfish samples for HEV (Gao et al., 2016). These developments are promising as the assays can be performed without the need for expensive equipment; however, a deeper characterisation and validation of these methods has not been performed so far.

Several commercial kits are available for HEV-specific RT-qPCRs. Most of these kits have been developed and validated for analysis of clinical samples from humans and only a few commercial assays intended for analysis of food and environmental samples are available.

3.2.1.3. Typing methods

Typing of HEV for the determination of the HEV genotype and subtype is usually performed by RT-PCR amplification of fragments of the HEV genome followed by sequencing and comparison with previously typed strains. Many different protocols for amplification of genome fragments have been developed. This includes protocols targeting the 5'-region of ORF1 (Preiss et al., 2006), the RdRp region of ORF1 (Johne et al., 2010) or the 5'-region of ORF2 (Meng et al., 1997; Schlauder et al., 1999; Preiss et al., 2006). The used primer pairs have also been adapted further in recent publications according to the increasing knowledge on the sequence variation (Vina-Rodriguez et al., 2015). Some of these protocols have been used more frequently than others, leading to an overrepresentation of HEV sequence fragments from distinct regions. Figure 2 shows the distribution of sequences available in the GenBank database according to their location on the HEV genome. According to this, sequences corresponding to the so-called regions ORF1c, ORF1d, ORF2e and ORF2f are mostly used, with a clear preference of ORF2e (boxed in Figure 2). These regions may be preferentially used in future for phylogenetic studies and HEV subtyping as they allow comparison with a large set of available sequences.

The sequence length used for typing largely influences the validity of the result. The use of whole genome sequences would clearly allow the best characterisation of a strain, including the assignment to a specific subtype. However, the capability for efficient generation and analysis of whole genome sequence data is still limited and expensive in many countries. Moreover, for some samples types, for example, for processed food samples, a low amount of the HEV genome and difficulties in its extraction and purification often restrict sequencing to short genome fragments. Vina-Rodriguez et al. (2015) suggest using sequences with more than 1,000 nucleotides for classification, whereas sequences shorter than 200 nucleotides should be avoided from subtyping. According to Lu et al.

(2006), the 5'-end of the ORF2 region better reflects the complete genomic sequence for HEV classification than other regions. In contrast, Vina-Rodriguez et al. (2015) show that partial sequences from the 5'-end of ORF1, the hypervariable region, RdRp region and ORF2 regions maintain the same basic tree topology as compared with whole genome sequences. However, reduced sequence lengths lead to lower percentage bootstrap supports and are therefore often not significant. Sequences from ORF3 are only partially suitable for classification up to subtype level, and the hypervariable region should be only used for intragenotype comparisons (Vina-Rodriguez et al., 2015). The hypervariable region is most divergent among different HEV strains, but very little variation is observed in this region among variants cocirculating within acutely infected individuals (Smith et al., 2012). Therefore, these regions could be successfully used for direct strain comparison in source attribution studies and outbreak investigations.

With the generated sequences, typing and subtyping are done by comparison to known sequences using methods for sequence alignments, nucleotide sequence distance calculation or phylogenetic tree construction. As different subgenomic regions, different reference sequences and phylogenetic methods are used for typing, some ambiguous results and incongruences have been reported in the past (Smith et al., 2014). Very recently, a distinct set of whole genome reference sequences for HEV-1 to HEV-7 subtypes has been proposed, which should strictly be used in further sequence comparisons and HEV subtyping (Smith et al., 2016). The list of reference strains is available online on the International Committee on Taxonomy of Viruses⁴ and will be updated as new data arises (Smith et al., 2016). In addition, attempts towards standardisation of HEV-3 subtyping have been made (Smith et al., 2015; Vina-Rodriguez et al., 2015). However, the classification of HEV strains into subtypes still suffers from different levels of diversity for the defined subtypes and from overlapping ranges of inter- and intrasubtype distances. Both distance-based and phylogenetic methods do not provide clear criteria for distinction between subtypes so far (Smith et al., 2015, 2016).

Recently, the European Centre for Disease Prevention and control (ECDC) has established an expert group of national public health epidemiologists and virologists to review the epidemiological situation of HEV in the European Union (EU)/EEA Member States (MS). The suggestion from this initiative was to develop a web-based sequence repository including a typing tool for HEV strains ('HEVnet') similar to HAVnet.⁵ 'HEVnet' will collect and analyse HEV sequence data and support the analysis with an embedded genotyping and subtyping tool. 'HEVnet' is based at the Dutch National Institute for Public Health and the Environment (RIVM) and was launched in 2017. The objectives include the public health support of HEV outbreak investigations and the assistance of epidemiological and clinical studies to better understand the underlying epidemiology related to circulating subtypes, clinical outcomes and trends. Related to the implementation of 'HEVnet', an exchange of knowledge and discussion among experts in the field is envisaged to agree on the harmonisation of typing and subtyping methods for HEV.

⁴ https://talk.ictvonline.org/ictv_wikis/hepeviridae/w/sg_hepe/343/proposed-orthohepevirus-a-subtype-reference-sequences

⁵ <http://www.rivm.nl/en/Topics/H/HAVNET>

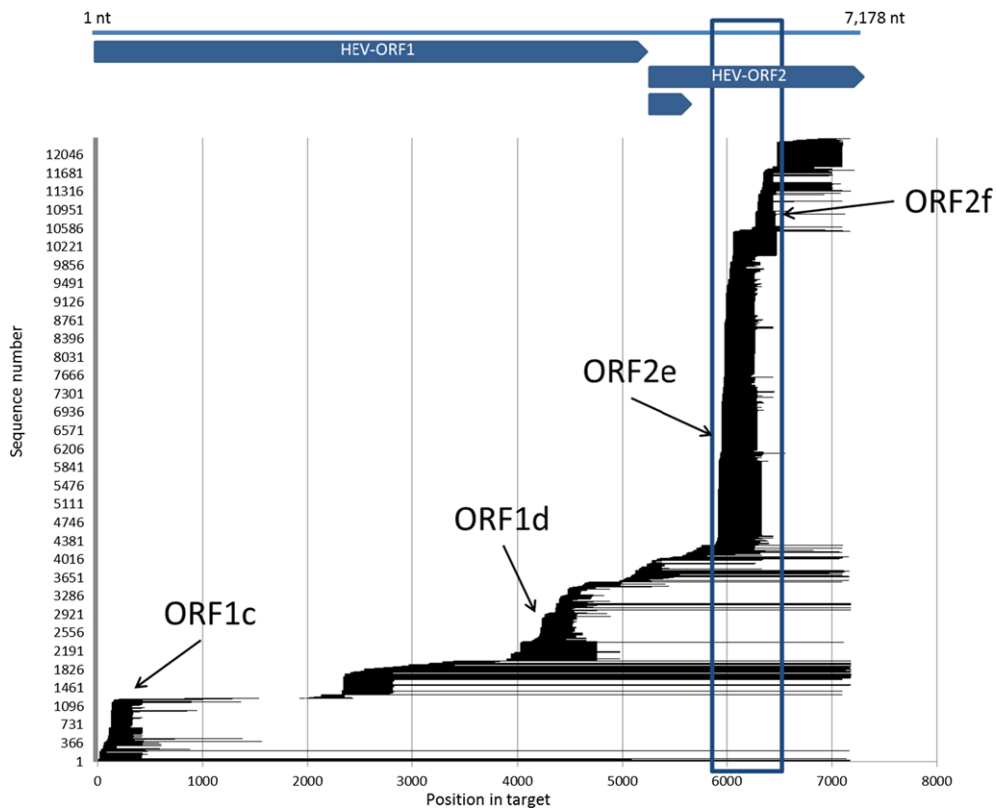


Figure 2: Distribution of sequences available in the GenBank (April 2016) database along the HEV genome (Courtesy of H. Vennema, RIVM)

3.2.1.4. Application of next-generation sequencing techniques

Although molecular assays (such as RT-PCR and RT-qPCR) provide sensitive detection, they are generally designed to identify only certain target viruses (Mengelle et al., 2013; Munro et al., 2013). Therefore, methods that do not depend on detection of limited nucleic acid (DNA/RNA) target sequences are becoming more important for identification of emerging viruses in a public health context. Utilising NGS, a high-throughput sequencing methodology that generates millions of sequences simultaneously from one sample, opened new perspectives for virus research and diagnostics. To date, several platforms have been developed; however, the two commonly used are Roche 454 pyrosequencing and Illumina (Shendure and Ji, 2008). The latest NGS systems (single-molecule real-time technology (SMRT)) are still in the evaluation stage and will soon be commercially available. The use of NGS techniques such as metagenomics should be considered (Wagner et al., 2016) for the characterisation of a community of contaminant viruses or identification of new viral strains, but further development is needed to improve the sensitivity of this as a pathogen detection method.

The applications of NGS allow reconstruction of full-length viral genomes (whole genome sequencing), and NGS deep sequencing produces valid analyses of viral genome variability (Lhomme et al., 2015a; Todt et al., 2016). As a method designed to analyse total nucleic acid (DNA/RNA) present in a given sample, NGS is suited to novel virus discovery and characterisation of viral communities (virome) in different hosts or environmental samples (Sachsenroder et al., 2012; Wang et al., 2015b), only a limited number of studies have been performed on food and food-producing animals.

HEV has only been characterised so far by NGS metagenomics-based strategies in untreated sewage as a source of environmental contamination (Ng et al., 2012), and during experimental HEV-3 cross-species infection (Bouquet et al., 2012). NGS-based whole genome sequencing studies focused on HEV in food and environmental matrices are currently hampered by the lack of virus amplification system (e.g. efficient cell culture), which could provide sufficient amounts of purified virus genomes. Therefore, NGS approaches directly from food and environmental samples need to be developed more to obtain full genome sequences.

3.2.2. Antibody-based assays

Antibody-based methods rely upon recognition of the specific immune response of the host organism against the causative agent. In the case of HEV in food-producing animals (animal reservoirs), the methods depend mostly on detection of anti-HEV antibodies belonging to the IgM and IgG isotypes against HEV-3 and HEV-4. The early production of IgM after infection, with its relatively short shelf-life, and the late development but longer duration of IgG (Meng et al., 1997; Takahashi et al., 2003) mean that IgM anti-HEV can be used as a marker for recent exposure. The presence of IgG anti-HEV is a sign of prior HEV infection and can be used to estimate the exposure to HEV in a population over a period of time. Anti-HEV antibodies can be detected by western blot (WB), enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA) formats. In the absence of a reference test assessing HEV antibodies, two tests, ELISA and WB, are recommended to use for human samples. In veterinary medicine, only one ELISA test is usually applied as a screening test (Thiry et al., 2014). The National Institute for Biological Standardization and Control provide reference material for quantitative detection of IgG anti-HEV (the concentration is defined in national units) in human sera. Recently, a veterinary serum standard has been developed to support quality control and comparison of antibody-based HEV tests (Zhang et al., 2017b).

Several commercial tests are available for detection of IgM or IgG in human sera. Because HEV-1 to HEV-4 were found in humans, these tests have been established using recombinant HEV-1, HEV-2 or HEV-3 ORF2- and/or ORF3-derived proteins, which were expressed in *Escherichia coli*, yeast, baculovirus-infected insect cells or mammalian cells. Alternatively, synthetic peptides or chimeric constructs harbouring multiple epitopes have been applied. Because it is believed that all mammalian HEV strains recognised to date belong to one serotype, the tests based on HEV-1 and HEV-2 should be able to confirm anti-HEV IgM and IgG against all HEV genotypes (Khudyakov and Kamili, 2011). However, the results of several studies showed variability in analytical sensitivity and specificity among commercially available assays, even for human sera (Pas et al., 2013; Wenzel et al., 2013; Echevarria, 2014). It has been also reported that anti-ORF2 antibodies tests based on HEV-1 are not absolutely specific, and thus they may be both less sensitive and less specific for detection of HEV-3 and HEV-4. Cross-reactivity of these tests has also been observed. Usually, false-positive results have been connected to the Epstein-Barr virus, cytomegalovirus, HAV, hepatitis B virus, autoimmune diseases or cancer (Worm et al., 2000; Waar et al., 2005; Bendall et al., 2010).

The availability of commercial assays for detection of IgM and IgG against HEV-3 and HEV-4 in food-producing animals is limited. Serological detection of anti-HEV antibodies in pigs and other animal species that can be infected with HEV can be performed by three methodologies: (1) commercially available assays (ELISA) for detection of specific anti-HEV antibodies in porcine serum; (2) commercial kits optimised for detection of human anti-HEV antibodies that have been adapted to use for swine or other animal species; and (3) in-house indirect or blocking ELISAs using HEV-3- or HEV-4-related ORF2 proteins as coating antigens (Krumbholz et al., 2013; Pezzoni et al., 2014; Ponterio et al., 2014; Van der Poel, 2014; Chen et al., 2016). Application of different serological assays also revealed some discordant results (Baechlein et al., 2010).

Krumbholz et al. (2013) tested randomly selected pig sera with five different tests. Three commercially available tests (based on recombinant ORF2- and ORF3-derived antigens of HEV-1 and HEV-3, on recombinant ORF2-derived antigen of HEV-1 and on recombinant capsid protein of HEV-3) and two in-house HEV-antibody ELISAs (based on rat HEV antigen and recombinant carboxy-terminal capsid protein derivatives of HEV-3). All three HEV-IgG tests based on HEV-3 proteins showed very high agreement (Cohen's kappa 0.757–0.824). Although HEV-1-based ELISA was found to give the highest degree of seropositivity, a markedly lower degree of agreement with HEV-3 proteins assays was observed (Cohen's kappa between 0.579 and 0.770). The majority of sera reacted only weakly or not at all with the rat HEV antigen, with very few sera showing as seropositivity as compared with the HEV-3 antigen.

A study from Zhang et al. (2011) compared three different commercial (porcine prototypes of human ELISAs) ELISAs, one in-house ELISA and one immunoblot test; all assays were based on different HEV proteins. Kappa analysis demonstrated substantial agreement (0.62–1.00) at 14 dpi, and complete agreement (1.00) at 56 dpi between the tests on samples from experimentally infected pigs. In contrast, an agreement (kappa value 0.182) of only 56.6% was found when using two different assays (HEV-1 peptide-based ELISA and HEV-1 recombinant protein-based commercially available ELISA) on field porcine samples with unknown HEV infection status. The results were mainly attributed to immunogenic differences between the proteins employed in the assays (Baechlein et al., 2010).

Various in-house indirect or blocking ELISAs and other EIA formats have been developed using coated antigens from HEV-3 and HEV-4 originating from pigs. The results indicated that in-house assays are at least as sensitive as the commercial ELISAs (Rose et al., 2010; Ponterio et al., 2014; Van der Poel, 2014).

Less frequently used is detection of IgA anti-HEV. IgA plays an important role in the immune function of mucous membranes and is elicited during the acute stage of different viral infections, including HEV infection. It can be considered as an alternative target to conventional IgM-based ELISA tests. The most common formats for IgA detection are in-house ELISAs. The role of the assays in conjunction with IgM anti-HEV in the diagnosis of acute infection has been explored in several studies (Takahashi et al., 2005; Elkady et al., 2007; Zhang et al., 2009). It was found that detection of anti-HEV IgA alone or with anti-HEV IgM (to detect more recent infections) is useful for serological diagnosis of current HEV infection with increased specificity and longer duration of positivity. However, it does not always reflect viraemia or virus shedding by faeces, which represents the main public health threat if present at slaughter (Takahashi et al., 2005; Crossan et al., 2015). The studies also suggest that some pigs do not have the ability to develop and maintain a detectable antibody level of IgM anti-HEV after HEV infection. Whether detection and quantification of IgA anti-HEV in pigs and other animals that may be natural reservoirs of HEV is useful as a tool to indicate likely viraemia or virus faecal shedding deserves further analysis (Takahashi et al., 2005; Khudyakov and Kamili, 2011).

As alternative method, using ELISA/EIA as a direct test for HEV antigen detection in serum or faeces has been described (Zhang et al., 2006; Zhao et al., 2015). The results of the test revealed significant correlation between EIA (S/CO) and HEV RNA (IU/mL) concentration in the range $10^{3.5}$ – $10^{0.5}$ IU/mL HEV RNA (the Pearson correlation coefficient r approached 0.97) in matrices tested. It showed lower sensitivity; the EIA detection limit was 54.6 IU/mL, compared with 24 IU/mL for HEV RNA using RT-qPCR (Zhao et al., 2015).

3.2.3. Infectivity assays

Methods for determination of HEV infectivity are either based on experimental inoculation of animals or on cell culture techniques. In addition, limited studies have been done using alternative molecular approaches, which aim to estimate infectivity. A recent publication presented a comprehensive literature overview on methods used for determination of HEV infectivity (Cook et al., 2016).

3.2.3.1. Inoculation of animals

Experimental inoculation of animals has been frequently carried out, and infectivity of HEV could be demonstrated in many studies using such *in vivo* models. However, most of these studies have been focused on determination of the host range of HEV and on clinical investigations, whereas only a few studies used animal inoculation for inactivation studies or infectivity determination of food samples. Clinical symptoms and elevation of liver enzymes indicative of hepatitis are almost exclusively described for infection of monkeys with high doses of HEV. In most of the studies using other animal species, including pigs, no signs of disease were evident after HEV infection. In these cases, faecal virus shedding, viraemia and/or seroconversion are used for identification of successful HEV infection (Cook et al., 2016).

Several animal species have been tested for susceptibility to experimental HEV infection. These include monkeys, pigs, rabbits, gerbils, rats, mice and tree shrews. While monkeys could be infected with HEV-1, HEV-2, HEV-3 and HEV-4, pigs were shown to only be susceptible to HEV genotypes 3 and 4 (de Carvalho et al., 2013; Johne et al., 2014a). Wild boars could also be infected with HEV-3 (Schlosser et al., 2014). Inoculation of rabbits and rats with HEV-1, HEV-3 and HEV-4 leads to divergent results, and some experiments resulted in infection and others not (Maneerat et al., 1996; Ma et al., 2010; Purcell et al., 2011; Cheng et al., 2012; Li et al., 2013). Mongolian gerbils have been successfully infected with HEV-1 and HEV-4 strains (Li et al., 2009; Yang et al., 2015; Soomro et al., 2016). Only single studies describe successful infections of immunodeficient mice (Huang et al., 2009) and tree shrews (Yu et al., 2016) using HEV-4 strains. Very recently, immunosuppressed mice repopulated with human liver cells have been developed as a human liver chimeric mice model for infection studies with HEV-1 and HEV-3 (Allweiss et al., 2016; Gouttenoire and Moradpour, 2016; Sayed et al., 2016; van de Garde et al., 2016).

Most inoculation studies have been performed with monkeys and pigs. In these animal species, different routes of infection have also been tested, and these indicate that intravenous infection is

most efficient, whereas oral infection usually failed. In an infection study with cynomolgus monkeys, an approximately 10^5 -fold higher dose of an HEV-1 strain was necessary to induce seroconversion by the oral infection route compared with intravenous inoculation (Tsarev et al., 1994). In a similar experiment with an HEV-3 strain in pigs, no seroconversion could be detected after infection using oral dosing or a stomach tube, whereas pigs intravenously inoculated with the same virus preparation readily seroconverted (Kasorndorkbua et al., 2002). In later studies, a low percentage of pigs orally inoculated with high doses of HEV showed seroconversion and/or faecal virus shedding indicating the possibility of oral infection; however, with low efficiency (Kasorndorkbua et al., 2004; Casas et al., 2009). Andraud et al. (2013) performed oral inoculation of pigs using various quantities of HEV genome equivalents (GE) (10^4 – 10^8 copies) to evaluate the oral infectious dose. None of the animals became infected (neither HEV shedding nor HEV seroconversion) with 10^4 and 10^5 GE, whereas two out of three and three out of three were infected with 10^6 and 10^8 GE, respectively (Andraud et al 2013).

Intravenous inoculation of pigs has been used for investigation of the presence of infectious virus in food samples. Feagins et al. (2007) inoculated pig liver suspensions derived from grocery stores in the USA, and reported seroconversion and faecal virus shedding in some of the animals. In contrast, Bouwknecht et al. (2007) observed no signs of infection in pigs inoculated with HEV RNA-positive livers from grocery stores from the Netherlands. A difference in the virus load of the inocula was discussed by the authors (Bouwknecht et al., 2007).

The swine bioassay using intravenous inoculation was successfully applied in inactivation studies of HEV-containing samples. This included experiments with heat-treated pig liver homogenates (Feagins et al., 2008) and heat-treated liver pâté-like preparations containing HEV-infected pig liver and other ingredients (Barnaud et al., 2012), which showed that application of sufficient heating protocols resulted in the absence of seroconversion in the inoculated pigs.

Although the animal inoculation models are generally useful for HEV infectivity assessment, some limitations are still evident. For instance, the amount of virus needed for infection of animals has not been specified in the publications as only the amount of HEV RNA has been specified. Therefore, the limit of detection and the possible range of live virus concentrations applicable for infectivity testing in the animal models are not known. Further limitations include ethical considerations as well as the restriction of sample numbers, because the experiments with large animals are laborious, time-consuming and expensive. Therefore, the use of animal inoculation as a method for detection and/or quantification of infectious HEV is limited (Cook et al., 2016).

3.2.3.2. Cell culture methods

A large number of publications describe successful isolation and propagation of HEV-1, HEV-2, HEV-3 and HEV-4 in cell culture (Okamoto, 2011, 2013; Johne et al., 2014a; Cook et al., 2016). In most cases, the human liver carcinoma cell lines PLC/PRF/5 and HepG2/C3A or the human lung carcinoma cell line A549 have been used in these experiments (Cook et al., 2016). However, other cell lines and cell lines derived from stem cells have also been successfully used (Rogee et al., 2013; Talbot et al., 2013; Helsen et al., 2016). HEV replicates in these cell lines generally slowly with increasing virus amounts for several days until it reaches moderate titres in the culture supernatant. In most of the studies, no cytopathic effect was evident due to HEV replication. Therefore, additional procedures like RT-qPCR or immunofluorescence are necessary to detect virus replication (Cook et al., 2016).

Recently, the HEV-3 strains Kernow-C1 and 47832c have been isolated from immunosuppressed transplant patients, which have been shown to replicate more efficiently compared with other field strains (Shukla et al., 2011; Johne et al., 2014b). Insertions in the hypervariable region of their ORF1 have been linked to the enhanced replication, although the distinct mechanism of action is not known. Also, subclonal cell lines derived from PLC/PRF/5 cells or A549 cells have been created, which showed different susceptibility to HEV (Shiota et al., 2015; Schemmerer et al., 2016). In addition, cell cultures have been grown in 2D and 3D format, and a higher sensitivity of the 3D grown cells for HEV was shown (Berto et al., 2013a,b).

Despite the numerous descriptions of successful HEV isolation, only a few studies tested the assays with regard to reproducibility and the detection limit. Generally, the HEV cell culture systems appear to have a high detection limit and varying reproducibility (Cook et al., 2016). Infectivity titrations over a range of different virus concentrations, which are needed for stability and inactivation studies, have only been described in a few studies using a limited set of cell culture-adapted HEV strains (Girones et al., 2014; Farcet et al., 2016; Johne et al., 2016). In detail, a system using HEV genotype 3 strain swJB-M5 and HepG2/C3A allowing a 3-log titration was applied in inactivation studies for blood

products (Farcet et al., 2016). The genotype 1 strain SAR-55 – concentrated by ultracentrifugation – could be titrated in a 3-log range using HepG2/G3 cells as described in a study assessing the effect of chlorine treatment (Girones et al., 2014). A titration in a 4-log range was described in a system using the genotype 3 strain 47832c in combination with A549/D3 cells for a heat stability study of HEV (Johne et al., 2016).

In most of the studies, HEV was isolated from clinical specimens such as serum, faeces or liver homogenates. Only two studies reported successful isolation of HEV from inoculated food samples. The first study describes HEV growth after inoculation of A549 cells with pig liver samples from retail (Takahashi et al., 2012), and the second study reports HEV replication after inoculation of PLC/PRF/5 cells grown in a 3D format with a liver sausage sample (Berto et al., 2013a). No standardised or validated method for preparation of food samples before inoculation into cell cultures is available, and further development and validation of such methods is required.

3.2.3.3. Alternative methods for infectivity assessment

Alternative methods that estimate infectivity by integrity testing of the capsid or the viral genome have been developed for several viruses. For noroviruses (NoV), a purification of intact virus capsids by binding to beads coated with receptor-like molecules has been used prior to RT-qPCR for estimation of infectivity (Dancho et al., 2012). Similarly, inoculation of cell cultures with the sample followed by RT-qPCR analysis of bound particles has been applied for these viruses (Li et al., 2014a). Treatment of NoV preparations with nucleases prior to RT-qPCR analysis has also been performed in order to enable exclusive measurement of RNA packaged into viral capsids (Mormann et al., 2010). In addition, long-range RT-PCR, which should exclude short and broken RNA fragments from RT-qPCR amplification, has been tested for infectivity estimation of NoV (Li et al., 2014a).

Only one study is available, in which an alternative method for infectivity estimation of HEV was developed (Schielke et al., 2011). In this study, the capsid integrity was assessed by RNase digestion before genome detection using RT-qPCR. The assay was used to estimate the thermal stability of HEV. However, a direct proof of the results by parallel infectivity testing using cell culture or animal inoculations has not been done.

Concluding remarks

- A diversity of methods for HEV extraction and RNA purification from animals and a range of food and water samples are available; however, standardisation and systematic method comparison and interlaboratory validation have not been performed for most virus extraction methods. Meat and meat products are considered to be high priority for method development.
- Quantification protocols for HEV, based on several (real-time) RT-PCR-based and LAMP-based techniques for the detection of the HEV genome, are available, although no standardised assays for use with animal, food or water samples are available so far.
- Several methods for typing and subtyping of HEV strains and for source attribution and tracing are available. However, the methods are not yet harmonised or standardised, leading to incongruences in subtyping of strains. The recent definition of HEV subtype reference strains, a distinct set of whole genome reference sequences for HEV-1 to HEV-7 subtypes proposed and the current development of the web-based typing tool 'HEVnet' represent important steps towards harmonisation. Thresholds for definition of types, subtypes and 'identical' strains based on sequence comparisons remain to be defined.
- The databases used in web-based typing platforms should be encouraged to also include strains from animals, food and environment in order to enable source attribution analyses.
- Several tests available for detection of IgM or IgG in human and animal sera have been established. However, as test characteristics vary between the different serological assays it is important to consider these when the results are interpreted.
- ELISA/EIA as a direct test for HEV antigen detection in serum or faeces as an alternative method correlates well with quantitative detection of HEV RNA in matrices tested, although it shows limited sensitivity.
- Numerous descriptions of successful HEV isolation in cell culture exist, although isolation from low HEV concentration clinical samples is often unsuccessful and only a few studies have tested the assays with regard to reproducibility and sensitivity. Reports on successful HEV isolation from food samples in cell culture are rare, and no standardised or validated method for preparation of food samples before inoculation into cell cultures is available.

- Pig or monkey inoculation models can be used for HEV infectivity assessment, including the analysis of food samples. However, the application of animal inoculation models is restricted by the unknown applicable range of virus concentrations, the limited potential for sample replication, ethical considerations, and the laborious, time-consuming and expensive nature of large animal experiments.
- Alternative methods for indirect estimation of HEV infectivity are so far restricted to a capsid integrity assay. The assay has not been validated by comparison with direct infectivity assays such as cell culture

3.3. HEV infection and disease in humans

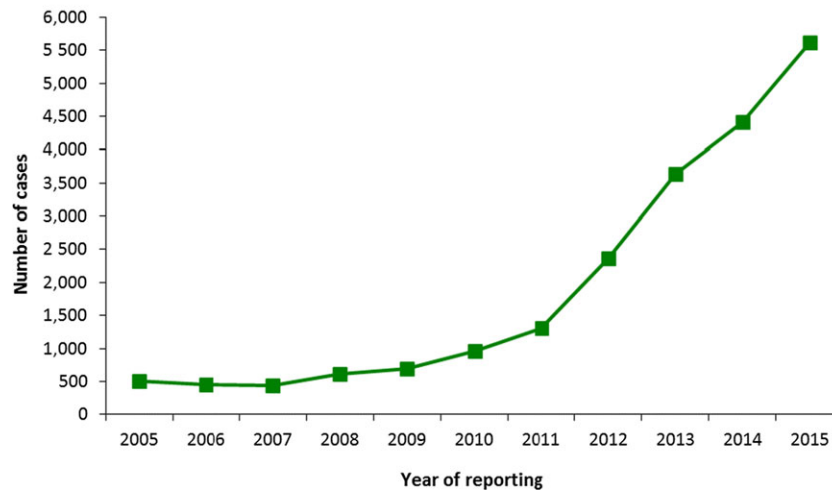
In Europe, HE is considered as an acute self-limiting hepatitis. The majority (> 70%) of infections are asymptomatic and people only seroconvert (Guillois et al., 2016). Symptomatic cases show an acute self-limiting hepatitis initially with fatigue, asthenia, nausea and fever. This can be followed by jaundice, with elevated liver enzymes, abnormal liver function tests (alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase, alkaline phosphatase and bilirubin), abdominal pain and hepatosplenomegaly. Clearance of infection is usually observed within 1–5 weeks, and the incubation period is estimated to be between 2 and 6 weeks (up to 60 days; Lhomme et al., 2016). HE diagnosis is confirmed by the detection of anti-HEV IgM in serum and/or HEV RNA in serum or stool samples.

3.3.1. Surveillance in EU/EEA and number of reported cases

HEV is not notifiable at the European level; however, notification requirement may be present in individual MS. Information is available about in which countries finding of HEV infection in humans is notifiable. Surveillance systems, case definitions and testing recommendations for target groups are under the authority of each MS. A recent study by ECDC collected information about surveillance systems, case definitions and number of cases reported in each country. Information was available for 30 countries, 67% (20/30) have national HEV-specific surveillance established and 10 countries reported to have a generic viral hepatitis syndromic surveillance in place (Adlhoch et al., 2016; ECDC, 2017). Ten countries have established a HEV-specific surveillance system since 2005 or earlier. A wide variation of case definitions for acute cases was observed across EU/EEA countries with a combination of clinical, laboratory and epidemiological definitions. Two countries also had a case definition for chronic cases in place.

The reported HE clinical cases increased from 514 in 2005 to 5,617 cases in 2015, with overall more than 21,000 cases reported from 22 countries between 2005 and 2015 (Figure 3). Several EU/EEA countries reported a considerable increase in human HE cases in recent years, which might be associated with increasing awareness amongst clinicians but also due to improved diagnosis, for example, inclusion of HEV in the standard hepatitis diagnosis panel (Ijaz et al., 2009, 2014; Pischke et al., 2014; Koot et al., 2015; Robert Koch Institut, 2015; Adlhoch et al., 2016). However, HEV is still considered to be an underdiagnosed disease in Europe. Some EU/EEA countries reported that the number of HE cases has been higher than the number of hepatitis A cases in recent years. Between 2005 and 2015 between 61% and 68% of all reported cases within EU/EEA were reported to be male. The proportion of cases being 50 years and older increased over the last 10 years from 30% to 61% in 2015. This confirmed that males above 50 years old represent the majority of acute cases of HEV infection reported across EU/EEA countries (Lewis et al., 2010; Adlhoch et al., 2016). As seen above (Figure 3), over the last 10 years an overall 10-fold increase in reported HEV cases in EU/EEA countries was observed; the majority (80%) of cases were reported from France, Germany and the UK. In recent years, 14 countries noted that more than 50% of the reported cases were hospitalised, although the proportion of hospitalised cases decreased over the last 10 years indicating a greater awareness of the disease in non-hospital settings. Between 2005 and 2015, 12 EU/EEA countries reported data on HEV-related deaths and five of these countries notified 28 deaths associated with HEV infection, increasing from 0–1 cases in 2005–2008 to 4–8 cases in 2010–2015. However, as infection in humans is not notifiable in all MS, and surveillance differs between countries, the number of reported cases is not comparable and the true number of cases would probably be higher.

Confirmed cases of Hepatitis E by year, EU/EEA Member States, 2005-2015*



*Data available for Austria, Belgium, Bulgaria, Croatia, Cyprus, the Czech Republic, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, the Netherlands, Norway, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden and the UK.

Figure 3: Number of reported human HE cases by year from 22 EU/EEA countries, 2005–2015 (ECDC, 2017)

More than 95% of the human infections were autochthonous, locally acquired (within the country of residence) due to infection with HEV-3 viruses, with the main subtypes being 3e, f, c, amongst others (Nelson et al., 2011; Abravanel et al., 2012; Zehender et al., 2014; Koot et al., 2015; Lapa et al., 2015; Smith et al., 2015). The number of travel-associated cases outside of EU countries remained at a low level over the years. Of a total of 15,525 human cases where information on travel related to HEV infection between 2005 and 2015 was available, only 240 (1.5%) have travelled to a country outside the EU/EEA and acquired the infection there (Figure 4).

Epidemiological studies identified eating pork or wild boar products, raw pig liver sausages or other processed pork products as risk factors for HEV infection in humans (Wichmann, 2008; Lewis et al., 2010; Said et al., 2014; Lucarelli et al., 2016). Vegetarians generally have lower seroprevalence; no vegetarians have been identified as cases in the UK study (Said et al., 2014).

No direct human-to-human transmission has been observed in the EU. Human-to-human transmission has not even been proven in large outbreak situations, for example, in refugee camps in high endemic countries where faecally contaminated drinking water was the source of infection (Hakim et al., 2017).

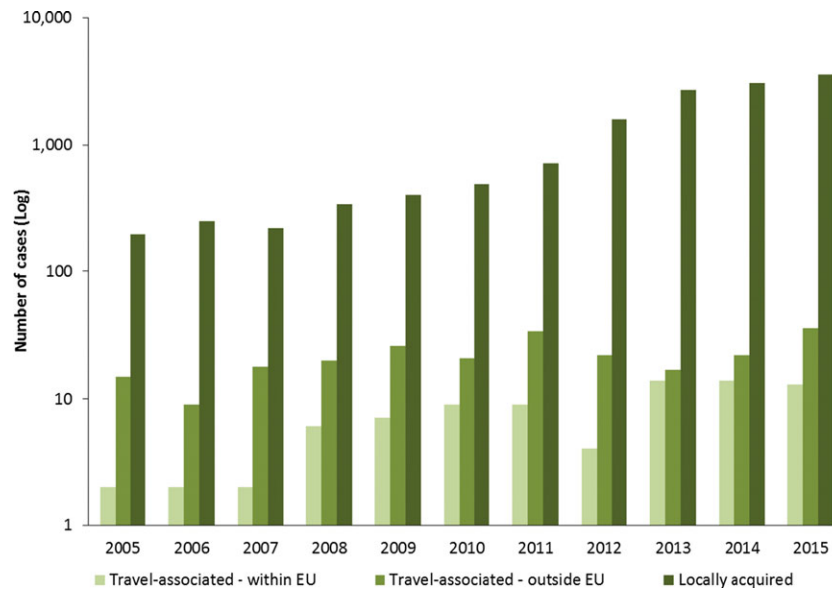


Figure 4: Number of human HE cases by origin of infection, 15 EU/EEA countries, 2005–2015 (ECDC, 2017)

3.3.2. HEV geno- and subtypes infecting humans in EU/EEA

In EU/EEA countries HEV-3 is the most prevalent virus genotype infecting humans. Only a few locally acquired (autochthonous, without travel to endemic countries during the incubation period) infections with HEV-4 viruses have been described in France, Italy and Germany. HEV isolates identified in French patients were similar to those detected in pigs in Belgium, but others matched those detected in pigs in China indicating that HEV detected in humans does not necessarily reflect the local epidemiological situation in the pig population, but global trade contributes to the dissemination of different geno- and subtypes across borders (Wichmann et al., 2008; Colson et al., 2012; Garbuglia et al., 2013; Jebblaoui et al., 2013).

In EU/EEA, genotype 3 viruses with the majority subtypes as HEV-3c and HEV-3e, f, g, and very few cases of HEV-4, have been described in humans. Sequences from viruses derived from pigs overlap in the phylogenetic tree with virus sequences from human cases who consumed pig meat. Molecular evidence of the source of transmission and relationship of viruses is achieved by studies that compared virus sequences and subtypes derived from human cases with viruses from pigs or consumed pork products. In the UK, replacement of predominant virus subtypes HEV-3e, f, g by HEV-3c in humans has been observed recently, while in the pig population in the UK, HEV-3e, f, g viruses are common, and this is thought to be related to imported pig meat or pork products from other EU countries where HEV-3c is prevalent in the pig population, for example, in the Netherlands, HEV-3c is found in human cases and the pig population (Ijaz et al., 2014; Koot et al., 2015). An increase of HEV-3c in human cases has also been observed in France recently, while HEV-3f decreased (Lhomme et al., 2015a). In Germany and Spain, HEV-3c and HEV-3f subtypes have been detected in human cases, and in pig or wild boar populations (Adlhoch et al., 2009a; Fogeda et al., 2009; Tabatabai et al., 2014; Wenzel and Jilg, 2014).

3.3.3. HEV seroprevalence in humans in the EU/EEA

Available data on seroprevalence for 19 European countries showed increasing seropositivity with increased age, and the highest values were observed in people above 60 years old (Hartl et al., 2016; ECDC, 2017). The studies showed different prevalence estimates across EU/EEA: Central European countries had higher seroprevalence than, for example, Nordic countries. Seroprevalence estimates differ not only between, but also within, countries, for example, the average national prevalence in the general population of Germany and France is about 20% (Faber et al., 2012; Mansuy et al., 2016), while in highly endemic regions (high incidences) due to the local traditional consumption of raw pork liver as in south France over 86% of the blood donors have tested HEV-positive; the same is true in Italy with more than 40% of the blood donors being HEV IgG seropositive in Central Italy while the national prevalence is reported to be 0.7–9.1% (Zanetti and Dawson, 1994; Vulcano et al., 2006;

Masia et al., 2009; Chaussade et al., 2013; Scotto et al., 2014; Lapa et al., 2015; Puttini et al., 2015; Hartl et al., 2016; Lucarelli et al., 2016; Mansuy et al., 2016; Tarantino et al., 2016). People occupationally exposed to pigs or wild boars, for example, working in the slaughterhouse, forestry workers, hunters, farmers or veterinarians, had a higher seroprevalence than the general population (Carpentier et al., 2012; Dremsek et al., 2012; Krumbholz et al., 2012; Chaussade et al., 2013; Hartl et al., 2015, 2016; Ivanova et al., 2015).

The difference in seroprevalence between and within countries could reflect different exposures of the population to the virus, for example, a lower or higher endemicity within the reservoir animals. Also, regional differences in consumer habits that result in higher risk of acquiring HEV infection have an impact on the seroprevalence, for example, through the consumption of contaminated raw pig liver products (Table 1). Variations in the sampling strategies, the population under surveillance and the use of different testing systems with varying sensitivity and specificity as well as other factors limit the comparability of the seroprevalence data across studies (Krumbholz et al., 2014; Hartl et al., 2016).

3.3.4. Chronic HE and HEV in immune-compromised and pregnant patients

Most humans with an acute infection recover completely within a couple of weeks. However, HEV infection in patients with pre-existing chronic liver disease can also lead to a fatal outcome due to liver failure (Kumar et al., 2008; Cheng et al., 2013). In African and Asian countries where HEV-1 and HEV-2 are endemic, pregnant women are under high risk for severe disease and fulminant hepatitis with liver failure, haemorrhage or eclampsia (Lhomme et al., 2016; Hakim et al., 2017). This can cause high mortality, estimated to be 21%, or premature delivery and infant mortality particularly when infections occur in the third trimester of pregnancy (Jin et al., 2016). In EU/EEA countries where HEV-3 is prevalent, the infection is not associated with severe disease in pregnant women and thus they are not considered as a risk group (Anty et al., 2012; Sánchez Díaz et al., 2012; Renou et al., 2013, 2014b; Tabatabai et al., 2014).

Persistent HEV replication can be observed in some patients, and persistence of the virus for more than 3 months is considered to be a chronic infection (Kamar et al., 2013). Patients with underlying chronic diseases or immunosuppressive conditions are at risk of developing chronic HEV infection with prolonged viraemia (> 6 months). These patients show limited symptoms of hepatitis or non-specific clinical symptoms, and can develop rapid liver cirrhosis with a fatal outcome (Page et al., 2011; Kamar et al., 2013; Pol, 2013). Patients with solid-organ transplantation, pre-existing liver disease or haematological malignancy are at increased risk for chronic disease development (Izopet et al., 2009; Tavitian et al., 2010; Nelson et al., 2011; Ijaz et al., 2014; Lapa et al., 2015; Hartl et al., 2016). However, immunosuppressive conditions might not in general be a risk factor for HEV infection and chronic progressive disease, for example, patients with common variable immunodeficiencies have not been identified as being at higher risk for HEV infection (Pischke et al., 2012). Also, HIV patients are considered as a risk group due to their compromised immune system, and some but not all studies show higher HEV seroprevalence, particularly among HIV-infected patients with low CD4 cell counts (cluster of differentiation 4) or cirrhosis (Mateos-Lindemann et al., 2014; Debes et al., 2016). Symptoms in these patients can vary from asymptomatic or acute hepatitis to liver failure. It has been speculated that this patient group may have increased risk of development of chronic disease, but results are discordant (Dalton et al. 2009; Colson et al., 2011; Mateos-Lindemann et al., 2014). Extrahepatic manifestations or chronic disease development in patients with HIV have not been noted with the same frequency as in other immune-deficient conditions (Debes et al., 2016).

The overall risk of developing chronic disease has been assessed as relatively low for Europe, taking the high risk of exposure into account (Renou et al., 2010; Pineda et al., 2014). The main routes of infection for such immunosuppressed patients are exposure to undercooked infected pork products, solid-organ transplantation and transfusion (Keane et al., 2012; Abravanel et al., 2014).

3.3.5. Transfusion and transplantation-related HEV infections

Transfusion or transplantation-transmitted HEV infections have been observed sporadically in the EU (Domanović et al., 2017). Although direct intravenous inoculation of contaminated blood products has been described, not all such events caused infections in humans. A minimum number of infectious particles is required for successful infection, and 20,000 IU has been identified as the lowest infectious dose for transfusion-transmitted infection via plasma products (Tedder et al., 2017). Two European countries (England and Ireland) have started selective or universal screening of blood donations, and others are considering implementation of such procedures (Domanović et al., 2017). The viral safety of

plasma-derived medicinal products regarding HEV has been addressed by the European Medicines Agency (EMA), and it was noted that transmission events have been observed for all blood components and, for example, an estimated 450 transfusion-transmitted infections per year result from contaminated blood components in the UK. It is assumed that widespread asymptomatic infection among blood donors is responsible for this.

Viraemia in blood or blood donations has been assessed as low to moderate, with titres seldom exceeding 10^6 IU/mL and with a frequency of viraemic donations ranging from 1:1,000 to 1:14,000. Experts from 11 European countries reviewed the data on transfusion or transplantation-related HEV transmission events, and stated that the main infectious risks for such immunosuppressed patients are dietary exposure to pork products and blood product transfusions (Domanović et al., 2017). In the last decade, an increasing incidence of HEV-3-positive donations has been documented in several European countries. Prevalence estimates in blood donors also show a regional- and age-specific distribution, showing higher values with increasing age. An overall increase of incidence in the general population, particularly in younger age groups, was observed by some countries such as Germany, the Netherlands and the UK with a higher number of HEV RNA-positive blood donors (Mansuy et al., 2008; Ijaz et al., 2012; Hogema et al., 2014; Wenzel et al., 2014; Tedder et al., 2016). Assessing the prevalence of HEV infection in pigs and pork meat consumption in the EU as well as the incidence of HEV viraemic blood donations, it was assessed that for the immunocompetent general population the risk of HEV transmission via food products is considerably higher than through blood transfusion (Müller et al., 2017). Further studies of infections associated with use of blood products or transplantation by means of seroconversion, sequencing of HEV genome using a standardised sequence database, and follow-up of HEV cases among blood donors and patients may help to define the relative contributions of different routes of HE infection in Europe. Addressing the issue of viraemic pigs entering the human food chain will be required to achieve control of HEV regardless of the secondary routes of dissemination (Domanović et al., 2017).

3.3.6. Extrahepatic manifestations of HE in humans

Hepatitis E infections do not only affect the liver but also have been described to have extrahepatic manifestations affecting several other organ systems, causing neurological symptoms, organ injuries or haematological disorders. HE infection has been associated with neurological disorders, such as Guillain-Barré, Parsonage Turner syndromes, neuralgic amyotrophy, bilateral brachial neuritis, peripheral neuropathy and encephalitis (Kamar et al., 2011, 2015; Madden et al., 2013; Van Den Berg et al., 2013, 2014; Scharn et al., 2014; van Eijk et al., 2014; Pérez Torre et al., 2015; Theochari et al., 2015; Dalton et al., 2016). Other extrahepatic manifestations are renal injuries, including membranoproliferative glomerulonephritis with or without cryoglobulinaemia and membranous glomerulonephritis, acute pancreatitis, and other autoimmune manifestations such as myocarditis, arthritis and thyroiditis (Kamar et al., 2012, 2015; Haffar et al., 2015). Thrombocytopaenia and other haematological disorders have also been observed (Fourquet et al., 2010; Woolson et al., 2014). Most of these presentations were described in solid-organ or bone marrow transplant recipients (Legrand-Abravanel et al., 2011; Pischke et al., 2011; De Niet et al., 2012; Abravanel et al., 2014).

3.3.7. Treatment and vaccination

No specific treatment is recommended for acute HE infection as it is considered a self-limiting disease. However, risk groups may require antiviral treatment, for example, people with pre-existing liver disease or immunosuppressed patients. In some cases, the reduction of immunosuppressive treatment supports the clearance of the virus. Antiviral therapy with ribavirin and in some cases pegylated interferon-alpha is indicated for treatment of chronic infections (Izopet et al., 2009; Izopet, 2010; Pol, 2013).

In December 2011, the first HEV recombinant subunit vaccine (Hecolin[®]) was registered in China. So far it has not been licensed and approved in other countries or territories (Wang et al., 2016). This vaccine is based on a 239-amino-acid-long recombinant HEV peptide, termed HEV 239, corresponding to amino acids 368–606 of open ORF2 of Chinese HEV-1 strain (Li et al., 2005). The vaccine protects against symptomatic HEV-4 infection, with a very high efficacy rate. The duration of follow-up in the available published reports has been for a period of up to nearly 2 years after administration of three doses over a 6-month period (0, 1 and 6 months); a limited study following that of the first two doses (at 0 and 1 month) was reported. Additionally, some unpublished data for up to 4 years after

completing immunisation are available. Long-term efficacy beyond this time point, duration of protection, and the need and timing for booster doses remain to be determined.

Random control trials have shown a high efficiency and very low number of serious adverse events following HE vaccination.

Due to gaps in knowledge, WHO does not recommend the introduction of the vaccine for routine use in national programmes, but national authorities may decide to use the vaccine based on the local epidemiology or in outbreak situations.^{6,7}

Concluding remarks

- Hepatitis E is an important infection in humans in EU/EEA countries causing more than 21,000 mostly locally acquired reported clinical cases with 28 fatalities over the last 10 years with an increasing trend. However, as infection in humans is not notifiable in all MS, and surveillance differs between countries, the number of reported cases is not comparable and the true number of cases would probably be higher.
- There are indications that the human population of Central European countries have a higher seroprevalence than, for example, Nordic populations.
- In EU/EEA, genotype 3 viruses with the majority subtype as HEV-3c and HEV-3e, f, g, and very few cases of HEV-4, have been described in humans. Molecular evidence of the source of transmission and relationship of viruses is achieved by studies that compare virus sequences and subtypes derived from human cases with viruses from pigs, wild boar and deer or products thereof.
- Immunosuppressed patients are at risk of developing chronic HEV infections with severe disease progression and fatal outcomes. A few human-to-human transmission events have been blood- and transplant-related.
- Occupationally exposed groups to the reservoir animals show higher seroprevalence than the general population, providing evidence for zoonotic transmission from animals to humans; vegetarian have a lower seroprevalence. Outbreak investigations and molecular studies identified the same viruses in the reservoir animal, food and infected humans
- In 2011, the first HEV recombinant subunit vaccine was registered in China based on a recombinant peptide HEV-1 strain, and has shown also to protect against symptomatic HEV-4 infection. Due to the lack of more information including efficiency against other genotypes so far, it has not been licensed and approved in other countries or territories.

3.4. Transmission of HEV from food and food animals to humans

This opinion is focused on transmission of HEV from animals, food and the environment. Humans and animals are the sources of HEV, food and environmental elements are vehicles within a transmission pathway.

Exposures and transmissions of HEV may vary from blood-borne, waterborne, food-borne or contact with animal reservoirs. The other transmission routes, such as person-to-person contact, are considered to be too inefficient to represent a significant risk of HEV transmission (Lewis et al., 2010). In the present section, evidence of food-borne transmissions will be described, including either sporadic cases or outbreaks. Level of proof was demonstrated using epidemiological indicators (e.g. shared meals in outbreaks) or meta-analysis studies and occasionally molecular epidemiology, showing the same HEV sequences in patients and the suspected food product.

3.4.1. Food-borne transmission of HEV in sporadic cases and outbreaks

Food-borne transmission can be divided between food made of raw material originating from an animal infected with HEV and food products contaminated with HEV. An example of the former is pig liver where the pig is infected with HEV. An example of the latter may be shellfish contaminated by water carrying virus from infected animals or humans (sewage). A recent national survey in France reported that risk factors associated to positive serology were: eating pork game or meat (RR 1.53), pork liver sausage (RR 1.30), oysters (RR 1.12) and offal (RR 1.25), while drinking bottled water was protective (RR 0.84; Mansuy et al., 2016). In Italy, the consumption of raw seafood, wild boar meat and liver sausage were identified as risk factors for locally acquired HEV infection (La Rosa et al.,

⁶ <http://www.who.int/wer/2015/wer9018.pdf?ua=1>

⁷ http://www.who.int/immunization/policy/position_papers/hepatitis_e/en/

2011). Consumption of pork products (pie, pâté, ham and sausages) was identified as a risk factor for HEV infection in the UK (Said et al., 2014). Indeed, indigenously acquired HEV has increased substantially in England and Wales since 2010. A case-control study was used to test the hypothesis that HEV infection is related to consumption of pork products. In a multivariable model, consumption of pork pies (odds ratio (OR) 6.3, 95% confidence interval (CI) 141–2848, $p = 0.009$), and consumption of ready-to-eat processed pork pies, ham and sausages purchased from a major UK supermarket chain (OR 10.1, 95% CI 168–6081, $p = 0.023$) were significantly associated with infection. The consumption of sausages and ham purchased from the supermarket was highly correlated; however, individual models showed that each variable was independently and significantly associated with infection (OR 7.6, 95% CI 181–3184, $p = 0.004$ and OR 10.98, 95% CI 184–6535, $p = 0.003$, respectively). Although contamination of sausages with HEV had previously been shown, several studies raise concerns about a variety of processed pork products (Hawkes, 2014; Said et al., 2014; Sarno et al., 2017). HEV infection of blood donors in the UK may be largely associated with consumption of uncooked ready to eat pig meat products from a particular retail chain (Tedder et al., 2016).

In a recent ECDC study collecting data on outbreaks of HE over the last 10 years, a total of 18 countries reported data on outbreaks, with 11 countries reporting no outbreak associated with HEV. Seven countries investigated a range of 0–3 outbreaks per year due to HEV in 2005–2010, and 4–8 outbreaks between 2011 and 2015, respectively. This apparent increase is possibly due, at least in part, to better diagnosis or reporting and/or awareness. The cumulative number of cases associated with these outbreaks ranged between 0 and 47 (Adlhoch et al., 2016; ECDC, 2017).

In the description of these food-borne HEV outbreaks, frequent association was found with the consumption of products containing raw or undercooked pig liver and also other pork products, such as pork pies, homemade sausages, undercooked or raw pork meat, processed pork products and offal (Deest et al., 2007; Colson et al., 2009, 2010; Berto et al., 2013a; Said et al., 2014; Guillois et al., 2016).

In 2013, three clustered clinical cases of HE infection (17 infections in total) were reported on a French coastal island. Individuals had consumed spit-roasted piglet that had been stuffed with a raw stuffing that included the liver of the piglet. Identical HEV strains to the human cases were detected in the liquid manure sampled at the farm where the piglet was born and in untreated human wastewater originating from communal sewage systems. Seventeen human infections associated with the meal were identified, of which 70.6% were asymptomatic. Symptomatic HEV infection among infected patients was independently associated with the quantity of piglet stuffing consumed (Guillois et al., 2016).

In another study, the consumption of grilled wild boar was shown to be responsible for HEV infection with the same HEV sequence found both in the patient and in the meat left over, which had been kept frozen (Li et al., 2005).

Besides pork products, consumption of deer meat has also been identified as a potential HEV threat in some countries (Saxena and Arora, 2014). Consumption of sashimi of sika deer origin was associated with a cluster of four cases with molecular data confirmation in Japan (Tei et al., 2003). The first case of acute HEV infection after ingesting raw wild roe deer meat was described in South Korea. Phylogenetic analysis of viral sequences from the patient's serum and leftover food identified HEV-4 (Choi et al., 2013).

Transmission of zoonotic HEV to humans via the consumption of seafood has not been proven directly yet as no identical or near-identical HEV sequences have been retrieved in patients suffering from HE and the seafood they had consumed. However, the consumption of shellfish has been strongly epidemiologically linked to an imported case of HEV-4 infection in a Japanese patient who consumed shellfish in Vietnam (Koizumi et al., 2004), and to a large HE outbreak on a cruise ship with 33 persons infected (HEV-3; Said et al., 2009).

A list of published outbreaks and sporadic food-borne cases in EU countries between 2005 and 2015 can be found in Table 1.

Table 1: Reported outbreaks and sporadic food-borne cases of HEV in EU member states for 2005–2015 (ECDC member state survey; ECDC, 2017)

Country	Year	Gt	Number of human cases	Vehicle/source of infection	Source unpublished	Reference
Austria	2015		2	Not identified	ECDC MS survey	
Czech Republic	2011		36	Tripe sausages made in farm and selling in butcher shop	ECDC MS survey	
Czech Republic	2009		1	Undercooked pork meat		Holub et al. (2008)
Czech Republic	2009–2011		2 outbreaks 13 and 8 cases	Unknown, transmission through consumption of pork and pork products at pig-slaughtering feasts assumed		Trmal et al. (2012)
Czech Republic	2009–2012		27 case reports	Possible factors (cases): pork meat (3), butcher, home pig slaughter (3), excessive pork intake, minced meat/liver sausage, brawn, liver sausage (3), raw pork meat, grilled pork meat, contact with HEV (2), greaves, minced meat, precooked sausage, home-made sausage, wild boar goulash, sausage		Chalupa et al. (2014)
France	2007		3	Raw figatelli	ECDC MS survey	
France	2009		1	Raw figatelli	ECDC MS survey	
France	2010		2	Raw figatelli		Renou et al. (2011)
France	2011		7	Unknown	ECDC MS survey	
France	2011		1	Probably raw figatelli		Anty et al. (2012)
France	2012		4	Unknown	ECDC MS survey	
France	2013		2	Unknown	ECDC MS survey	
France	2013	3	17	Undercooked pig liver-based stuffing in spit-roasted piglet	ECDC MS survey	Guillois et al. (2016)
France	2015		7	Private well connected to public water supply	ECDC MS survey	
France	2007		2	Likely dried pig meat		Deest et al. (2007)
France	2014	3	1	Likely raw pig liver sausage (figatelli)		Doudier et al. (2015)
France	2011–2012	4	4	Raw pork liver sausage, not thoroughly cooked pig meat		Colson et al. (2012), Tesse et al. (2012)
France	2011	3	1	Raw pig liver sausage (figatelli)		Moal et al. (2012)
France	2013	3	2	Raw pig liver sausage (figatelli)*		Renou et al. (2014a)
France	2007–2009	3	7	Raw pig liver sausage (figatelli)		Colson et al. (2010)
Germany	2006		2	Unknown	ECDC MS survey	
Germany	2008		2	Unknown	ECDC MS survey	
Germany	2009		2	Unknown	ECDC MS survey	
Germany	2011		2 outbreaks 4 cases	Unknown	ECDC MS survey	
Germany	2012		3 outbreaks 6 cases	Unknown	ECDC MS survey	

Country	Year	Gt	Number of human cases	Vehicle/source of infection	Source unpublished	Reference
Germany	2013		2 outbreaks 4 cases	Unknown	ECDC MS survey	
Germany	2014		3 outbreaks 8 cases	Unknown	ECDC MS survey	
Germany	2015		6 outbreaks 14 cases	Unknown	ECDC MS survey	
Hungary	2012		2	Unknown	ECDC MS survey	
Hungary	2014		2 outbreaks 4 cases	Unknown	ECDC MS survey	
Hungary	2015		2	Unknown	ECDC MS survey	
Hungary	2004	3	1	Home-prepared pork sausage		Reuter et al. (2006)
Italy	2011	3	1	Likely figatelli from France*		Garbuglia et al. (2015)
Italy	2011	4	5	Unknown		Garbuglia et al. (2013)
Spain	2014	3	1	Pork meat*		Riveiro-Barciela et al. (2015)
Spain	2015	3f	8	Wild boar meat*		Rivero-Juarez et al., (2017)
UK	2008		33	Shellfish		Said et al. (2009)

HEV: hepatitis E virus; ECDC: European Centre for Disease Prevention and Control; MS: Member State.

*: Molecular confirmation of the source/vehicle.

Unknown: epidemiological link between cases evident, no food item identified.

3.4.2. Transmissions through direct contact with infected animals

As mentioned previously, persons working in the slaughterhouse, forestry workers, hunters, farmers or veterinarians have a higher seroprevalence than the general population, thus direct and indirect contact with infected animals presents a risk for HEV exposure (Lange et al., 2017). Contact with infected pigs or their organs (pet pig, surgery training, slaughterhouse worker) was reported as a confirmed source of HE, and was recorded in three reports (Colson et al., 2007; Perez-Gracia et al., 2007; Renou et al., 2007), but living in a pig-dense area where farm biosecurity and waste management is carefully controlled was not associated with increased risk (van Gageldonk-Lafeber et al., 2017).

In a study in France, serum samples were collected from 859 healthy subjects, including pig farm workers, forestry workers and people without contact to animals, considered to be a control population. Anti-HEV antibodies were detected in 26% of the control population, and in 36% and 44% of forestry and pig farm workers, respectively. Consumption of pork liver sausage (AOR 4.4, $p < 10^{-4}$), occupational contact with animals such as wild boar (AOR 1.58, $p = 0.038$ for forestry workers and AOR 2.51, $p < 10^{-4}$ for pig farm workers), and living in southern France (AOR 1.47, $p = 0.02$) were independent risk factors (Chaussade et al., 2013). Although wild boars may present a risk to a small proportion of the population, regular occupational exposure to wild boars and their habitats and frequent consumption of wild boar meat is not common.

Concluding remarks

- Food-borne transmission appears to be the major pathway for human HEV infections in Europe. Raw or undercooked pork meat or pork liver sausages are the most frequently reported food products associated with sporadic cases or outbreaks of HEV.
- Domestic pigs are the main animal reservoirs of HEV in the EU. Wild boars are also an important reservoir, but their population is lower compared to pigs and wild boar meat is less commonly consumed.

- Contact with infected animal reservoirs is also a risk for HEV. Personnel with professional occupation with pigs or wild boar are more frequently exposed than the general population.

3.5. HEV occurrence in animals

Domestic pigs are the main animal reservoirs of HEV worldwide. Anti-HEV antibodies have also been detected in a large range of other animal species, including wild boars, deer, moose, rats, dogs, cats, mongooses, cows, sheep, goats, avian species, rabbits, bats and horses. Transmission from animals to humans is documented through direct and indirect evidence in many countries, but many animal species carry strains of HEV that are unrelated to those associated with zoonotic infection (Montalvo Villalba et al., 2013; Pavio and Bouquet, 2014; Lin et al., 2015; Khuroo et al., 2016; Roth et al., 2016).

Table 2 shows the results of various investigations about the occurrence of HEV in the major food and wild game animal species that have resulted in zoonotic transmissions in the EU. Most investigations have involved fairly limited local or regional surveys or convenience sampling in certain countries, so results may not be representative of the national prevalence. Methodologies and sample types used are also variable, both for serological and viral detection as well as molecular characterisation.

Table 2: Summary of investigations into the occurrence in the EU of HEV in the major domestic and wild game animal species that have been implicated in zoonotic infections

Animal species	Country	Year	Animal age	Number of tested animals	Type of sample	% Positive samples by serological tests (type of antibodies)	% Positive samples by RNA detection (genotype)	Reference	
Pigs	Croatia	2009–2010	Not specified	848	Blood, spleen, liver	N.D.	24.5% ^{d,l,m} (HEV-3)	Pripic et al. (2015)	
	Estonia	2013	Adult	380	Serum	61.6% ^a	–	Ivanova et al. (2015)	
		2013	1.9–4 months	449	Faecal samples	–	22.9% ^{l,m} (HEV-3)	Ivanova et al. (2015)	
	France	2009–2010 (national survey)	Slaughter-aged animals	6,565 3,715	Sera	31% ^b	4% ^{l,m} (HEV-3); 76.7% (HEV-3f) 18.6% (HEV-3c) 4.6% (HEV-3e)	Rose et al. (2011)	
					Livers	–	–	–	–
	Germany	2011	6–9 months	120	Faecal samples at slaughter	N.D.	2.5% (HEV-3i)	Machnowska et al. (2014)	
	Germany	2011	3–9 months	796	Serum	38.4% ^{c,*}	N.D.	N.D.	Krumbholz et al. (2013)
					Serum	51.4% ^{c,*}	N.D.	N.D.	Krumbholz et al. (2013)
					Liver	N.D.	13.5% ORF-1 primer; 7.6% ORF-2 primer; all HEV-3	Baechlein et al. (2013)	
	Ireland	2010–2011	Breeding animals	330	Serum	27% ^c	N.D.	O'Connor et al. (2015)	
Italy	2008	> 9 months	111	Serum	92% ^c 94% ^d 73% ^e	N.D.	N.D.	Ponterio et al. (2014)	
				Blood	80% ^a	N.D.	N.D.	Costanzo et al. (2015)	
				Faecal	N.D.	7.4% ^l	Costanzo et al. (2015)		
Netherlands	2004	6 months (at the slaughter)	130	Serum	68% ^f	N.D.	Rutjes et al. (2014)		

Animal species	Country	Year	Animal age	Number of tested animals	Type of sample	% Positive samples by serological tests (type of antibodies)	% Positive samples by RNA detection (genotype)	Reference
Pigs	Spain	2009	Not specified	48	Serum	43.8% ^c	0% ^{n,m}	Kukielka et al. (2016)
	Switzerland	2006, 2011	< 1 year old	2,001	Serum	58.1% ^c	N.D.	Burri et al. (2014)
		Not specified	Fattening animals	160	Liver	N.D.	1.3% ^o	Müller et al. (2017)
	UK (national survey)	2013	Slaughter-age animals	629	Serum, plasma	92.8% ^c	3.0% ⁿ	Grierson et al. (2015)
		2013	Slaughter-age animals	629	Caecal content samples	N.D.	15.00% ⁿ	Grierson et al. (2015)
		2006	Slaughter-age animals	176	Serum	61.4% ^c , (IgG, 29%; IgA, 36.9%; IgM, 29%)	44.4% ^{l,m} (HEV-3)	Crossan et al. (2015)
	Lithuania	2014–2015	Weaned pigs – adults	384	Serum	43.6%	ND	Spanceriene et al. (2017)
	Norway	1994, 2009, 2010	Not specified (archived samples)	663	Serum frozen faeces	73%	HEV RNA found in faeces from 3 herds	Lange et al. (2017)
	Croatia	2009–2010	Not specified	536	Blood, spleen, liver	N.D.	12.3% ^{d,l,m} (HEV-3)	Prpic et al. (2015)
	Czech Republic	2009–2013	Wild	190	Bile, liver, faeces	N.D.	18.42% ⁿ (HEV-3)	Kubankova et al. (2015)
2009–2013		Farmed	260	Bile, liver, faeces	N.D.	23.46% ⁿ (HEV-3)	Kubankova et al. (2015)	
Estonia	2013	Not specified	471	Meat juice	17.2% ^a	16% of Ab ^{+,l,m} (HEV-3)	Ivanova et al. (2015)	
France	2009–2010–2012	Young, < 14 months; subadult, 15–25 months; adult, > 26 months	376	Serum, liver	29.2% 95% CI = 24.5–34.4%	2.3% 95% CI = 1.0–4.6%	Jori et al. (2016)	
Wild boar								

Animal species	Country	Year	Animal age	Number of tested animals	Type of sample	% Positive samples by serological tests (type of antibodies)	% Positive samples by RNA detection (genotype)	Reference	
Wild boar	Germany	2013–2014	Not specified	48 sera, 95 sera/liver	Sera, liver	27.1% ^g	6.3% ^{n,#}	Anheyer-Behnenburg et al. (2017)	
		2014–2015	Not specified	132 sera, 137 liver/sera	Sera, liver	51.5% ^g	24.1% ^{n,#}	Anheyer-Behnenburg et al. (2017)	
		2013–2015	Not specified	141	Sera, liver, muscle, spleen, kidney		100% ⁿ liver 82.9% ⁿ muscle 85.2% ⁿ spleen 84.2% ⁿ kidney 94.1% ⁿ serum	Anheyer-Behnenburg et al. (2017)	
			2011	Not specified	330	Serum	33% ^h	N.D.	Denzin and Borgwardt (2013)
			2007	1 year old (n = 53), 1–2 years old (n = 38), adults sows (n = 21), adult boar (n = 9), undetermined (n = 11)	132	bile (n = 119), blood sample (n = 115), liver (n = 126)	29.9% ^{i,**} 26.2% ^s	68.2% ^{n,m} overall 15.7% ^{n,m} serum 56.3% ^{n,m} bile 38.1% ^{n,m} liver (all HEV-3, subtypes: HEV-3i, HEV-3h, HEV-3f, HEV-3e)	Adlhoch et al. (2009b)
			2012–2014	All ages	372	Liver	N.D.	1.9% ⁿ (HEV-3e, HEV-3c, HEV-3f)	Serracca et al. (2015)
			2012–2013	All ages	594 (serum), 320 (liver)	Serum, liver	4.9% ^c	3.7% ^{k,i,m}	Caruso et al. (2015a)
			2011–2012	Young (strikes, n = 2), subadult (no strikes, weight < 20 kg; n = 32), adult (n = 30)	64	Serum, faecal sample	56.2% ^j	9.4% ^k faecal sample	Nardini et al. (2014)
			not specified	0–12; 13–35; > 35 months	Serum (n = 228), liver (n = 164)	Serum and liver	40.7% ^c	33.5% ^k (HEV-3)	Montagnaro et al. (2015)

Animal species	Country	Year	Animal age	Number of tested animals	Type of sample	% Positive samples by serological tests (type of antibodies)	% Positive samples by RNA detection (genotype)	Reference
	Netherlands	2005–2008	4–18 months	1,029	Faeces, liver, muscle (diaphragm)	28% ^f	8% ⁿ	Rutjes et al. (2010)
	Portugal	2011–2013 2011–2012	Farmed Juveniles (6–14 months old), young adults (15–25 months old), mature adults (> 26 months)	40 80	Faecal samples Liver	N.D. N.D.	10% ^l (HEV-3e) 25% ^l (HEV-3e)	Mesquita et al. (2014) Mesquita et al. (2014)
	Slovenia	Not specified	Various ages	288	Serum	30.2% ⁱ	0.3% ^k	Zelev et al. (2016)
	Spain	2003–2010	< 6 months (n = 8), 7–12 months (n = 35); 12–24 months (n = 39); > 2 years (n = 68)	108 serology, 158 RNA detection	Serum	57.4% ^c	10.1% ^{n,m}	Kukielka et al. (2016)
	Lithuania	2014–2015	Juvenile–adult	312	Serum	57.1%	N.D.	Spanceriene et al. (2017)
Wild boar	Switzerland	2008–2012	< 1 year old (n = 124), subadults (n = 83), ≥ 2 years old (n = 91)	303	Serum	12.5% ^c	N.D.	Burri et al. (2014)
Deer	Croatia	2009–2010	Not specified	320	Blood, spleen, liver	N.D.	0% ^{k,l}	Prpic et al. (2015)
	Czech Republic	2009–2014 2009–2014	Wild Farmed	237 36	Liver, faeces Liver, faeces	N.D. N.D.	1.3% ⁿ 0% ⁿ	Kubankova et al. (2015) Kubankova et al. (2015)
	Italy	2012–2014	Not specified	30	Liver	N.D.	0% ⁿ	Serracca et al. (2015)

Animal species	Country	Year	Animal age	Number of tested animals	Type of sample	% Positive samples by serological tests (type of antibodies)	% Positive samples by RNA detection (genotype)	Reference
Germany		2000–2001/ 2011–2013	Various ages and species of hunted wild deer	145 sera/100 sera, 102 whole blood, 101 liver	Serum, blood liver	2.0/3.3% (red deer), 5.4/6.8% (roe deer)	2.0/6.6% (red deer) 4.3% (fallow deer)	Neumann et al. (2016)
				31 sera, 34 liver/sera	SERUM, LIVER	0 ^j	0.06% ⁿ	Anheyer-Behmenburg et al. (2017)
		2014–2015	Not specified	128 sera, 139 liver/sera	Serum, liver	0 ^j	0.04% ⁿ	Anheyer-Behmenburg et al. (2017)
		2013–2015	Not specified	24	Serum, liver, muscle, spleen, kidney		80.0% ⁿ liver 100.0% ⁿ muscle 50.0% ⁿ spleen 50.0% ⁿ kidney 60.0% ⁿ serum	Anheyer-Behmenburg et al. (2017)
Netherlands		2006–2008	0–2 years	46	Faeces, liver, muscle (diaphragm)	8.7% ^f	15% ⁿ	Rutjes et al. (2010)
Spain		2003–2010	< 1 year old (n = 2), 1–2 years old (n = 5), 2–3 years old (n = 6), > 3 years (n = 68)	70 serology, 81 RNA detection	Serum	12.9% ^c	16.05% ^{n,m}	Kukielka et al. (2016)
Lithuania		2014–2015	Roe deer–various ages	166	Serum	1.2%	ND	Spanceriene et al. (2017)

N.D.: not done; HEV: hepatitis E virus.

Serological methods: ^acommercial HEV Porcine Ab ELISA kit; ^bcommercial HEV Human Ab ELISA kit; ^ccommercial HEV Ab ELISA kit; ^din-house ELISA; ^ewestern blotting; ^fMPB HEV dasELISA; ^gHEV-specific IgG ELISA; ^hHEV3 antibodies; ⁱELISA; ^jHEV-specific IgG ELISA; ^k3 different ELISA kits were used; ^l**two different ELISA kits were used. RNA-detection methods: ^kdirect RT-PCR; ^hnested RT-PCR; ^msequencing; ⁿnot specified; ^otest performed on liver and sera.

3.5.1. Pigs

HEV infection in pigs is considered to be subclinical, but low-grade transient hepatitis has been reported after experimental infection (Tang et al., 2016). The level of viraemia and faecal shedding of the virus at slaughter is variable according to the management system, the age at first exposure and at slaughter and opportunities for recycling of infection, but HEV RNA is still detectable in some tissues in the absence of serum HEV RNA in both swine HEV- and human HEV-inoculated pigs, suggesting the possibility infection or contamination via mechanisms other than current circulation of HEV in blood. Replicative, negative-strand HEV RNA can be detected primarily in the small intestines, lymph nodes, colon and liver (Williams et al., 2001).

In a statistically designed and fully randomised national survey of pigs in France in 2009–2010, the seroprevalence and prevalence of HEV-positive livers in slaughter pigs was assessed; 6,565 sera and 3,715 livers were randomly sampled from 186 pig farms throughout the country. The calculated farm-level seroprevalence was 65% (95% CI 57–74), and 31% (95% CI 24–38) of the slaughter-aged pigs had anti-HEV antibodies. The prevalence of HEV RNA-positive livers was 4% (95% CI 2–6), and 24% (95% CI 17–31) of the farms were associated with at least one positive finding in a liver. Most HEV were subtype 3f (76.7%), with some 3c (18.6%) and 3e (4.6%) HEV (Rose et al., 2011). In a later full national survey of multiple pathogens in the UK in 2012, HEV antibody prevalence, active infection and virus isolation in serum and caecal content samples from 629 pigs at slaughter was investigated. The prevalence of antibodies to HEV was 92.8%, and HEV RNA was detected in 15% of caecal contents, 3% of plasma samples and 2% in both sample types (14/629; Grierson et al., 2015). In other smaller surveys, seroprevalence ranged from 27% in a study of 330 Irish breeding pigs in 2010/2011 (O'Connor et al., 2015) to 94% of old stored serum samples from Italian slaughter pigs (Ponterio et al., 2014). HEV RNA detection ranged from 0% in 48 serum samples tested in Spain in 2009 (Kukielka et al., 2016) to the 15% cited above for UK caecal samples.

There is evidence that HEV can be vertically transmitted in pigs and disseminated through the breeding pyramid. RNA from sera, islet and other cells from a population of 40 miniature pigs set up for breeding for xenotransplantation were examined for HEV using different RT-qPCRs. In addition, sera were examined by WB using two recombinant capsid proteins of HEV as antigens. HEV RNA was not detected in pigs older than 1 year including gilts, but it was detected in the sera of 3 out of 10 animals younger than 1 year. Furthermore, HEV was also detected in the sera of three sows 6 days after delivery and in their piglets, suggesting vertical transmission. The viruses were found to belong to genotype HEV-3 and HEV-4. Anti-HEV IgG was detected in one sow, and maternal antibodies in her 6-day-old piglet (Morozov et al., 2015).

Shedding of HEV was detected in 87.5% of piglets during a study of 11 farms in Finland. Piglets on breeding farms became detectably HEV-positive 3–8 weeks after weaning and, by the time they were transferred to fattening farms, virtually all (96.6%) pigs shed the virus. The HEV sequences obtained belonged to genotype 3, subtype e, and a unique, farm-specific strain was associated with 10 out of 11 farms examined. Trade with HEV-infected pigs promotes HEV transmission from farrowing to fattening farms (Kantala et al., 2015).

In pig farms, dynamics of infection are variable according to management systems, with late infections occurring shortly before slaughter-time presenting a greater risk due to HEV presence in liver. The transmission of this enteric virus between pigs is strongly influenced by environmental faecal contamination (Kasornrondkubua et al., 2004), which suggests the possibility to reduce the prevalence of infected pigs by appropriate farm management, hygiene and biosecurity measures (Rose and Pavio, 2014).

The dynamics of transmission in pig herds must be elucidated to reduce the prevalence of viraemic pigs at the time of slaughter that are produced by endemically infected herds, which are likely to be the majority of herds in many countries. Improved knowledge of vertical transmission and other routes of spread between herds is also required. An experimental trial was carried out in France to study the main characteristics of HEV transmission between orally inoculated pigs and naive animals. A mathematical model was then used to investigate transmission routes: direct contact between pigs and environmental contamination. A large inter-individual variability was observed in response to infection with an average latent period lasting 6.9 days (5.8; 7.9) in inoculated animals and an average infectious period of 9.7 days (8.2; 11.2). The results showed that direct transmission alone is a factor in persistence of infection within groups of pigs. However, the quantity of virus present in the environment was also found to play an important role, strongly influencing the probability of infection, particularly within groups rather than between separate pens. Between-pen environmental

transmission was also important as it led to spread within the farm and further within-group dissemination. The combination of these transmission routes that occur commonly on pig farms could explain the persistence and high prevalence of HEV in pig populations (Andraud et al., 2013).

In another French study on a single farm, HEV RNA testing was performed on rectal swabs sampled in 2012 from 50 × 3-month-old pigs from the same farm that was sampled in previous work during 2007. Prevalence by RT-PCR was similar to that determined 5 years earlier (68% vs 62%). Two distinct phylogenetic clusters of HEV subtypes 3a and 3f were identified, but the sequences obtained in 2012 largely differed compared with those obtained in 2007. Notably, HEV sequences obtained in 2012 from the majority (62%) of the infected pigs belonged to subtype 3a, which had not been previously described in France (Colson et al., 2015).

Genetic divergence and transmission of HEV was investigated among pigs at a Finnish breeding pig testing station on two occasions. In 2007, HEV RNA was found in 25% of pens, and 35% of 2–3-month-old pigs at the station. Three different HEV clusters, comprising 13 sequences of HEV genotype 3e in pigs imported from different farms, were detected. In 2010, 39% of pigs were HEV RNA-positive on weeks 1, 3 or 5 of a 3-month follow-up, and 11 sequences, all representing one of the HEV that was also present in 2007, were detected. The virus was considered to be either re-introduced, or to have persisted at the premises and was transmitted between pigs (Kantala et al., 2013).

Spray-dried porcine plasma in pig feed

Spray-dried porcine plasma (SDPP) is an ingredient commonly used in feed for weaned pigs in many countries around the world. SDPP is manufactured from blood collected from commercial pig-slaughtering facilities. It is a high-protein ingredient that provides nutritional and immunological benefits for newly weaned piglets (Van Dijk et al., 2001).

It is estimated that in the spray drying process the porcine plasma is normally heated to about 80°C. However, it is very hard to estimate the temperatures reached in the core of the particles and the variability in different parts of the drying cabinet under varying environmental temperature conditions.

Even though processing conditions used to produce SDPP should theoretically be sufficient to inactivate HEV, this requires confirmation in real-life situations. A small convenience study to analyse commercial SDPP samples for the presence of genome and antibodies against HEV (to assess potential exposure to HEV), and to retrospectively analyse serum samples collected from pigs used in past experiments that had been fed diets containing either 0% or 8% SDPP to detect potential transmission of HEV, as determined by seroconversion was set up to investigate this. Eighty-five commercial SDPP samples were analysed by ELISA, and 100% of them contained antibodies against HEV, while 22.4% of 49 samples were positive for HEV RNA. This shows that viral RNA is present in the material but the viability and infectivity is unknown, and there is no information on the risk of HEV transmission. Reported studies to date have not identified evidence that feeding SDPP in diets for pigs is likely to represent a risk of transmitting HEV, even though HEV genome may be detected in SDPP (Pujols et al., 2014), and this is also suggested by observations relating to heat treatment of human plasma products (Farcet et al., 2016), but such studies have been small, non-representative and lacking statistical power, therefore larger more carefully designed studies are required to assess the potential risk across the large output of this diverse industry, including an assessment of the risk of intermittent suboptimal heat treatment that may occur under certain environmental conditions.

3.5.2. Other animals

3.5.2.1. Wild Boar

In Europe and the Far East, food-borne zoonotic transmission of HEV-3 has been associated with domestic pigs and wild boar (Okano et al., 2014). In a controlled transmission study, HEV-3 infection was detected in wild boar and miniature pigs kept in contact with intravenously inoculated wild boar. High virus loads and prolonged viral shedding by wild boar present an important HEV risk (Schlosser et al., 2014).

For wild boar, seroprevalence ranged from 4.9% of 594 samples tested in Italy in 2012/2013 (Caruso et al., 2015a) to 56.2% of 64 samples tested in Italy in 2011/2012 (Nardini et al., 2014). HEV RNA was detected in 1.9% of 373 Italian wild boar liver samples between 2012 and 2014 (Caruso et al., 2015a), increasing to 33.5% of 164 hunted wild boar liver samples in another local survey in Italy (Montagnaro et al., 2015).

3.5.2.2. Deer

High IgG seroprevalence is found in deer in regions with high wild boar densities but prevalence is low in red deer farms, even though stocking density is high, but where red deer have no contact with wild boar or domestic swine. Wild boar densities are also high in fenced hunting estates, and increased HEV antibody prevalence is reported in associated deer. These observations could suggest that red deer may often need an external source of infection and thus act as spill-over hosts rather than true reservoirs (Boadella, 2010).

In deer, seroprevalence ranged from 2% to 3.3% in red deer in Germany across various years (Neumann et al., 2016) to 12.9% of 70 samples in Spain (Kukielka et al., 2016). HEV RNA was detected in 0 of 320 spleen and liver samples in Croatia in 2009/2010 (Prpic et al., 2015), and in 16.1% of 81 samples from red deer in Spain (Kukielka et al., 2016).

3.5.2.3. Rabbits

HEV isolated from rabbits is genetically distinct from strains detected in most human HE cases, and has been found in farmed rabbits in several geographic areas of China, USA and France. HEV RNA was detected in 7% of bile samples from farmed rabbits and 23% of liver samples from wild rabbits in France in one study and 5% in another (Lhomme et al., 2015b). Sequence analysis indicates that all the strains from rabbits belonged to the same clade, which represents a distinct subtype closely related to HEV-3. Comparison with HEV sequences of strains circulating in France identified one strain from a human case that was closely related to HEV found in rabbits and which was able to cause infection in a pig model. HEV from rabbits can also replicate efficiently in human cell lines (Lhomme et al., 2013). In a more recent study in France, of 919 HEV-infected patients during 2015–2016, five immunosuppressed patients were retrospectively found to be infected with a rabbit HEV strain. None of the patients had direct contact with rabbits or had eaten rabbit that was not well cooked, suggesting the possibility of another food-borne or waterborne infection route (Abravanel et al., 2017). In another study, phylogenetic analysis of HEV found in farmed, wild and pet rabbits in the Netherlands showed them to be grouped among published rabbit HEV sequences and distinct from most HEV detected in human samples. Dutch rabbits are therefore considered unlikely to be a significant zoonotic source (Burt et al., 2016). Some HEV strains from rabbits appear to have close sequence homology with some strains found in people (Izopet, 2012). The virtually identical results obtained in genetic analyses suggest that rabbit and human HEV ORF2 antigens contain very similar immunodominant epitopes (Wang et al., 2013c) and are likely to cross-react in serological investigations (Wang et al., 2013b).

HEV originating from farmed rabbits in China has been shown experimentally to be able to infect both cynomolgus macaques and pigs, and HEV found in pigs can infect rabbits (Han et al., 2014a; Liu et al., 2017). It was concluded that although interspecies transmission of rabbit HEV can be achieved experimentally, in the field it is not likely to be a significant factor in the epidemiology of HEV (Han et al., 2014b).

A serological survey for HEV infection in rabbits was conducted in Italy during 2013–2014, including both farmed and pet rabbits. Anti-HEV antibody seroprevalence was 3.4% in 206 farmed rabbits (collected on seven farms) and 6.6% in 122 pets. RNA was extracted from IgG-positive sera and tested by HEV-specific RT-qPCR. None of the samples was positive. Only one serum sample from a farmed rabbit was positive for IgM, but no HEV RNA was detected. Pet rabbit faeces were also tested for HEV RNA, with negative results (Di Bartolo et al., 2016). A HEV strain that is closely related to strains detected in humans has been identified in a 7-year-old pet rabbit (*Oryctolagus cuniculus*) in Italy (Caruso et al., 2015b).

3.5.2.4. Domestic ruminants

In a USA study, 16% of 80 goat sera from Virginia milking herds were positive for IgG anti-HEV, and neutralising antibodies to HEV were present in selected IgG anti-HEV-positive goat sera. However, a prospective study in a closed goat herd with known anti-HEV seropositivity, which monitored 11 kids from the time of birth until 14 weeks old for evidence of HEV infection, identified IgG anti-HEV in seven of the kids, although repeated attempts to detect HEV RNA by a broad-spectrum nested RT-PCR from faeces and serum samples from the seropositive goats were unsuccessful. In addition, attempted experimental infection of laboratory goats with three well-characterised mammalian strains of HEV was unsuccessful (Sanford et al., 2013).

In Nigeria, a serological study of pigs (67), goats (43), sheep (19) and cattle (37 for HEV antibodies IgG and IgM) using ELISA revealed an overall HEV seropositivity of 24.1% with IgG and IgM positivity of 16.3% and 7.8%, respectively. Goats recorded the highest prevalence; 37.2%, followed by pigs at 32.8% and sheep with 10.5%, but no positive reactions were found in cattle (Junaid et al., 2014).

In Egypt, HEV seropositivity was recorded in 21.6%, 14%, 4.4% and 9.4% from cows, buffaloes, sheep and goats, respectively. The infected food animals were closely associated with HEV-positive human populations (El-Tras et al., 2013).

One-hundred and eighty goat serum samples collected in the Panxi area of China were tested by ELISA for antibodies against HEV: 26.11% (47/180) were positive, of which 19.26% (26/13) were in large goat farms and 46.67% (21/45) in small-scale goat production ($p < 0.01$), and younger goats were more likely to test positive (Deng, 2014).

A study of HEV infection in Chinese sheep involved 500 sheep sera and 75 raw sheep liver samples from a slaughterhouse, along with 26 sera of workers from the same slaughterhouse. The results indicated that sheep seroprevalence (by ELISA) was 35.2% and that four of the 5.3% sheep livers showed detectable amounts of HEV RNA by RT-PCR. The seroprevalence amongst abattoir workers was 57.7%. The four strains from sheep liver formed a lineage within a genotype 4 cluster, all of which belonged to genotype 4, subtype 4d (Wu et al., 2015).

Although antibodies to HEV have been reported from immunodiagnostic studies of cattle in some countries, evidence of active infection is so far confined to China (Ruggeri et al., 2013). In one Chinese study, a high prevalence of active HEV infection was found in dairy cows, as determined by viral RNA shedding in faeces. HEV was also excreted into milk produced by infected cows. All HEV detected in cows/milk belonged to HEV-4. Inoculation of rhesus macaques with HEV-contaminated raw or pasteurised milk from an infected cow resulted in HEV shedding, as assayed by PCR, but a short period of boiling of milk completely inactivated HEV and there was no transmission of infection (Huang et al., 2016). Surprisingly, the inoculated monkeys did not seroconvert, and more supportive data are required to confirm the results of this study. Very recently, HEV-4 sequences have also been detected in samples from yellow cattle in China (Yan et al., 2016).

3.5.2.5. Moose

Serum samples from 231 moose were taken in seven Swedish counties, and the HEV prevalence determined by RT-PCR and serological assays of sera as well as 51 faecal samples. Sixty-seven animals (29%) were positive by one or both assays, while 34 (15%) were positive for HEV RNA, 43 (19%) were seropositive for anti-HEV antibodies, and 10 (4%) had both markers. The proportion of anti-HEV antibody-positive individuals increased with age. The sequenced moose HEV genome was only 35–60% identical to existing HEVs. Partial ORF1 sequences from 13 moose strains showed high similarity among them, forming a distinct monophyletic clade with a common ancestor within the HEV genotype 1–6 groups (Lin et al., 2015). It appears that the Swedish moose HEV strains are not closely related to those currently found in humans, unlike those occurring in wild boar, and are likely to be species-specific (Meng, 2016; Roth et al., 2016). In Lithuania, 11.8% of 34 serum samples from moose were found positive for anti-HEV antibodies during a study of domestic pigs and wild game mammals conducted between 2014 and 2015 (Spancerniene et al., 2017).

3.5.2.6. Camels

Dromedary camel HEV (DcHEV or HEV-7), a novel HEV, has been identified in dromedary camels in the United Arab Emirates. An antigenic analysis demonstrated that HEV-7 was cross-reactive with HEV-1, HEV-3–HEV-6, ferret and rat HEVs, showing stronger cross-reactivity to HEV-1 and HEV-3–HEV-6 than to rat and ferret HEV. In addition, the antibody against HEV-7 neutralised HEV-1 and HEV-3 in a cell culture system, suggesting that the serotypes of these HEVs are identical (Woo et al., 2014; Zhou et al., 2015). A large study screened 2,438 dromedary camel samples from Pakistan, the United Arab Emirates and four African countries. HEV-7 seems to be long established, diversified and geographically widespread in camelids (Rasche et al., 2016).

Very recently, a putative new HEV genotype (HEV-8) has been identified in 3 of 305 faecal samples of Bactrian camels from China (Woo et al., 2016).

3.5.2.7. Rats

Wild rats can be reservoirs of numerous zoonotic pathogens. Screening of rat samples from Germany with a broad-spectrum RT-PCR led to the identification of rat HEV, a virus distantly related to HEV-1 to HEV-7 and recently grouped into the genus *Orthohepevirus* C (Johne et al., 2010; Smith

et al., 2014). Meanwhile, rat HEV has been shown to be widely distributed in different rat species worldwide (Johne et al., 2014a). The zoonotic potential of this virus seems to be low, although rat HEV-specific antibodies have been recently demonstrated in a small number of human samples (Dremsek et al., 2012).

In contrast to the wide distribution of rat HEV, detection of HEV-3 in rats has been described only once (Lack et al., 2012). In this study, HEV-3 RNA was detected in liver tissues from 34 of 446 rats from the USA. Efforts to infect laboratory rats with HEV-3 were not successful, whereas injection of transcripts of a HEV-4 cDNA into the liver of rats led to transient seroconversion (Johne et al., 2014a).

3.5.2.8. Marine mammals

Bottlenose dolphins housed at the National Aquarium, Havana, Cuba, some of which were suffering from liver disorders, were evaluated for HEV. Anti-HEV antibodies were detected in 32.2% (of the 31 dolphins tested), and 16.1% of the dolphins were positive by both serology and HEV RNA testing. Nucleotide sequence analyses revealed that HEV strains identified in dolphins were HEV-3. The source of infection is uncertain but may represent an environmental contamination of food or water, and it is speculated that this may also occur in the wild through consumption of contaminated shellfish (Villalba et al., 2017).

3.5.2.9. Birds

Avian HEV is a virus associated with big liver and spleen disease or hepatitis-splenomegaly syndrome in chickens, and subclinical or non-specific clinical infections by the virus are also common and widespread (Johne et al., 2014a; Zhao et al., 2017). This virus is distinct from HEV-1 to HEV-7, and has been recently grouped into the genus *Orthohepevirus* B (Zelenika et al., 2013; Zhao et al., 2013; Smith et al., 2014; Gerber et al., 2015; Yugo et al., 2016). Infection experiments by intravenous inoculation of avian HEV into rhesus monkeys did not result in seroconversion, viraemia or faecal virus shedding indicating the absence of a zoonotic risk of avian HEV (Huang et al., 2004).

In contrast to the frequent detection of avian HEV, only two publications report the identification of HEV-3 and HEV-4 in birds. HEV-3 was detected in two Himalayan griffons housed in a zoo in China (Li et al., 2015a). HEV-4 was detected in a crowned crane and a silver pheasant from a zoo-like location in China (Zhang et al., 2008). Recently, similar genotypes to some of those found in chickens have been identified in wild birds (particularly pigeons, but also buzzard, little owl, song thrush) in Europe (Zhang et al., 2017a).

Recently, a divergent HEV was amplified in birds of prey, common kestrel (*Falco tinnunculus*) and red-footed falcon (*Falco vespertinus*) in Hungary (Reuter et al., 2016). It is distant from all *Orthohepevirus* species (Figure 2).

Concluding remarks

- Domestic pigs and wild boars are the most important HEV reservoirs, and also the species most frequently investigated.
- HEV infection of pigs is largely subclinical, but minor hepatitis has been reported after experimental infection. The duration of viraemia and faecal shedding of the virus is variable according to the management system, the age at first exposure and opportunities for recycling of infection.
- Transmission of HEV from infected sows to piglets and trade in carrier pigs has been responsible for wide dissemination of infection. Specific HEV strains can persist for long periods on pig farms, but a turnover of strains can also occur.
- Infected animals are carriers of the virus that can be shed at a high level in faeces and bile, and can be found at a lower level in meat. This could represent a possible vehicle for food cross-contamination during slaughter, evisceration and food processing, including processing in the home.
- HEV-infected pigs have been identified at the farm and abattoir level in all countries where investigations have been carried out. Prevalence of HEV varies greatly between farms, production systems and countries, with small non-industrialised production being at higher risk in some studies.
- A proportion of pigs, likely to be less than 10%, remain viraemic at slaughter, which is a probable cause of prime meat cuts containing HEV.
- Studies in some countries suggest that HEV-free pig herds may exist, but this should be confirmed by more intensive testing.

- Cumulative genetic and epidemiological evidence from several countries where wild boars are prevalent suggests that HEV infection is common and poses a zoonotic hazard for hunters and consumers of wild boar meat.
- Deer meat may present a zoonotic risk; however, the HEV prevalence in deer is comparatively low. There appears to be a statistical association between the prevalence of HEV in wild boar and deer populations in some regions.
- A few host-associated HEV strains of rabbits (HEV-3ra) appear to have close sequence homology with some strains found in people but the relative importance of natural transfer of HEV from rabbits to people needs to be determined. Antibodies to HEV and RNA have been found in farmed, wild and laboratory rabbits in several countries, but rabbits appear to be resistant to infection with HEV-3 strains detected in humans and pigs.
- There is no evidence of HEV infection in goats, sheep and cattle in Europe. However, specific antibodies to HEV have been found in other countries, especially in areas where there is heavy environmental exposure to HEV. More data are required to clarify the reservoir status of these animal species for zoonotic strains of HEV.
- HEV-7 has been detected once in an immune-compromised patient exposed to camels. The zoonotic potential of HEV-7, and a proposed HEV-8, and the risk of transmission from camel to humans remain to be elucidated.
- Various animals, such as moose, rats, ferrets, bats and several species of birds, have been reported to carry host-specific variants of HEV and there is currently no evidence for zoonotic transmission.

3.6. HEV occurrence and persistence in food

There is direct (detection of HEV RNA) and epidemiological evidence that supports the link between the onset of HE and the consumption of HEV-contaminated food of animal origin, causing both sporadic cases and outbreaks of infection. Domestic pigs and wild boars are the most important reservoirs and also the species most investigated.

This chapter summarises most of the studies conducted on HEV RNA detection in organs from animals for food production, as well as in food of animal (e.g. including mussels, which are not infected but contaminated by HEV) and non-animal origin. HEV RNA has been detected over the entire production chain of pigs (farms, slaughterhouse, processing and retail, and in several other animal reservoirs also intended for food consumption; Doceul et al., 2016).

3.6.1. Liver and liver products

In Europe, HEV RNA was detected in pig liver collected during slaughter, with prevalence varying among countries (Doceul et al., 2016) and ranging between 1.3% (2/169) in Switzerland (Müller et al., 2017) up to 13.5% (34/251) in north-western Germany (Baechlein et al., 2013). The study conducted by Di Bartolo et al. (2012) involving Spain, the Czech Republic and Italy, reported no evidence of liver faecal cross contamination during slaughter (Di Bartolo et al., 2012).

In non-EU countries, HEV RNA detection in pig liver was reported in Canada (20.9%; 9/43; Leblanc et al., 2010), in China (6.3%; 6/95; Li et al., 2011b), 1.7% (2/118) in Brazil (Cardinali et al., 2012), and the highest prevalence was reported in Colombia (41.3%; 62/150; Gutierrez-Vergara et al., 2015) and China 16/51 (Hong Kong, 31%; CFS, 2010). In the latter study, HEV-4 was detected.

In the UK, the Netherlands, Germany, India, Thailand, Colombia, Canada and USA, HEV RNA has been detected in pig liver sold in grocery stores and markets, with a prevalence of 1.3% (1/76), 6.5% (4/62), 4% (8/200), 0.83% (2/240), 0.27% (3/1090), 25% (25/100), 8.8% (25/283) and 11% (14/127), respectively (Bouwknegt et al., 2007; Feagins et al., 2007, 2008; Kulkarni and Arankalle, 2008; Banks et al., 2010; Wenzel et al., 2011; Wilhelm et al., 2014; Gutierrez-Vergara et al., 2015; Intharasongkroh et al., 2016).

HEV RNA was also detected in liver from wild boar in Europe (Table 3), in China (6.3%, 6/95) (Li et al., 2011b) and in Japan (3.4%, 19/552) (Sato et al., 2011).

Several papers have reported HEV RNA detection in pork sausages and pork liver sausages (Doceul et al., 2016). In Spain, the UK and Germany, 6% (6/93), 9.5% (6/63) and 26% (13/50), respectively, of pork sausages sold in the market were positive for HEV RNA (Berto et al., 2012; Di Bartolo et al., 2012; Szabo et al., 2015). HEV RNA has been investigated and detected in some of these specialties, pork liver and non-liver products sold in several European countries, such as figatelli 30% (42/140; PAVIO et al., 2014), dried salted liver (3% 1/30), quenelle and quenelle paste (25%, 13/55), dried or fresh liver sausages (29%, 49/169) in France (PAVIO et al., 2014); raw (22.2%, 10/45) and dry (4.3%,

1/23) liver sausages in Italy (Di Bartolo et al., 2015); liver sausages (22%, 11/50) and raw wild boar sausage (10%, 1/10) in Germany (Szabo et al., 2015); in chitterlings (non-liver pork products, 25% 3/12) purchased in southwest Virginia (Cossaboom et al., 2016). HEV replication was demonstrated in 1 out of 4 HEV-positive pork liver sausages obtained from France, by using a 3-dimensional cell culture system. This paper proved the persistence of infectious viruses in processed food (Berto, 2013 #20).

3.6.2. Meat

Few papers report detection of HEV RNA in meat (muscle); 1/22 (4.5%) in wild boar hunted in central Germany (Schielke et al., 2015), in red deer diaphragm muscle (5%, 2/39) in the Netherlands (Rutjes et al., 2010), and in 1/40 and 2/33 pigs lingual muscle analysed in the Czech Republic and in Italy, respectively (Di Bartolo et al., 2012). In Thailand, 0.36% (2/559) pork samples from fresh markets (for retail sale to consumers, where liver, meat and other animal products were sold together) were positive for HEV (Intharasongkroh et al., 2016). In pork products sold in Brazil (Heldt et al., 2016), different formulations of pâté were positive for HEV RNA (ham 11/36, bacon with meat 6/6), while bacon (2 tested), liver (1 tested) and knuckle with cheese (2 tested) as well as blood sausage (2 tested) were negative. In animals that have been tested HEV positive in the liver (and muscle samples were also available), HEV RNA was detected in 29/35 (82.9%) muscle samples in the case of wild boars and in 6/6 (100%) muscle samples in the case of deer. However, the HEV RNA load was consistently lower in muscle samples as compared with liver samples (Anheyer-Behmenburg et al., 2017).

No HEV RNA was detected in 28 packages of rabbit meat purchased in Southwest Virginia (Cossaboom et al., 2016).

3.6.3. Milk

Milk as a possible source of HEV transmission has recently been suggested in a paper describing a patient from the Middle East, who regularly consumed milk from an infected camel (Lee et al., 2016). However, no information is available on HEV occurrence in camel milk in general. Detection of infectious HEV-4 in milk samples from 52 HEV-infected cows has been reported in China (Huang et al., 2016) while, in another study, HEV RNA was not detected in 400 milk samples from cattle of unknown HEV status, collected in 2008 from dairy farms in Germany. Nevertheless, as suggested by the authors, in China the cows were sampled in a rural area where mixed farming of domestic animals is a common practice, in contrast with the industrialised farms of cows analysed in Germany that had no contact with other animals (Baechlein and Becher, 2017).

3.6.4. Shellfish

HEV, as an enteric pathogen of both humans and animals, is shed in faeces and is therefore present in wastewaters and coastal waters. Bivalve molluscan shellfish are known to concentrate viruses during the process of filter feeding and so they may accumulate HEV.

HEV-3 or HEV-4 RNA was detected in shellfish, such as oysters and mussels from Galicia (14.81%, 12/81; Mesquita et al., 2016), bivalve molluscs from the Netherlands (4%, 3/84 oysters and 3/84 mussels; Pol-Hofstad et al., 2014), oysters from Korea (8.7%, 14/161; Song et al., 2010), bivalves harvested in rivers in Japan (6.25%, 2/32; Li et al., 2007), and shellfish from the coastal waters of China (17.5%, 22/126; Gao et al., 2015). A high prevalence (85%) was observed in 48 mussels for human consumption collected in different areas of Scotland. One collection area was around an outfall (drain/sewage pipe) directly in line with a pork-processing plant considered by the authors to be a potential source of contamination (Crossan et al., 2012).

A study conducted in Spain, Greece and Finland, evaluating the presence of enteric viruses in 153 commercial mussels, reported 6% (3/51) of positive samples in Spain only and no significant correlation between the presence of human adenovirus and NoV, HAV and HEV (Diez-Valcarce et al., 2012). Several studies reported no detection of HEV in shellfish, including samples positive for human enteric viruses (La Rosa et al., 2010; Iaconelli et al., 2015; Fusco et al., 2017).

Experimental bioaccumulation studies and an investigation conducted using mussels as biomonitors have shown that oysters, mussels and clams can concentrate HEV. Mussels and clams were found to be more sensitive to sporadic contamination events, as demonstrated by rapid bioaccumulation in less than 1 h, compared with oysters for which concentrations increased over 24 h (Grodzki et al., 2014).

Table 3 provides an overview of studies reporting HEV RNA detection in organs and food of animal origin.

Table 3: Occurrence of HEV RNA detection in organs for food production and in food of animal origin, in Europe

Country	Animal species	Place	Sample	No. of HEV RNA-positive/total (%)	RT-qPCR, log ₁₀ copies (range/g)	Reference
Belgium (Walloon Region)	Wild boar	Hunting	Liver	4/61 (6.5)	–	Thiry et al. (2015)
	Red deer		Liver	1/29 (3.4)		
France (Corsica)	Pig	Slaughterhouse	Liver	2/24 (8.3)	–	Jori et al. (2016)
France (South-western)	Deer	Hunting	Liver	2/62 (3.2)	2.78 (1.11–3.07)	Lhomme et al. (2015b)
	Wild rabbit		Liver	1/20 (5)	8.7	
	Wild boar		Liver	5/86 (5.8)	2.80 (1.57–8.05)	
France	Pig	Retail	Figatelli Dried salted liver quenelle and quenelle paste Dried or fresh liver sausages	42/140 (30) 1/30 (3) 13/55 (25) 49/169 (29)	2.23–5.83 5.83 2.41–5.45 2–6.36	Pavio et al. (2014)
France	Pig	Slaughterhouse	Liver	128/3,715 (4)	–	Rose et al. (2011)
France (South-Eastern)	Wild boar	Hunting	Liver	7/285 (2.5)	–	Kaba et al. (2010)
Germany	Wild boar	Hunting	Liver and/or serum	39/232 (16.8)	WB: 7.35 Deer: 3.35	Anheyer-Behmenburg et al. (2017)
	Roe deer			5/78 (6.4)		
	Red deer		Muscle from animals HEV-positive in liver	2/83 (2.4)	WB: 3.64 Deer: 2.72	
	Wild boar			29/35 (82.3)		
	Roe deer			4 (100)		
Red deer	2 (100)					
Germany (Central)	Wild boar	Hunting	Liver Muscles	4/22 (18) 1/22 (4.5)	–	Schielke et al. (2015)
Germany	Pig	Retail	Raw sausages	13/50 (26)	–	Szabo et al. (2015)
	Wild boar		Liver sausages	11/50 (22)		
	Wild boar		Raw sausages	1/10 (10)		
Germany (North-western)	Pig	Slaughterhouse	Liver	34/251 (13.5) and 19 (7.6)*	9	Baechlein et al. (2013)
Germany (South eastern)	Pig	Grocery store	Liver	8/200 (4)	6	Wenzel et al. (2011)
Germany (East and West)	Wild boar	Hunting	Liver	48/126 (38.1)	–	Adlhoch et al. (2009b)
Germany (Federal states of Brandenburg and Thuringia, cities of Berlin/Potsdam)	Wild boar	Hunting	Liver	22/148 (14.9)	–	Schielke et al. (2009)

Country	Animal species	Place	Sample	No. of HEV RNA-positive/total (%)	RT-qPCR, log ₁₀ copies (range/g)	Reference
Hungary	Wild boar	Hunting	Liver	8/75 (10.7)	–	Forgach et al. (2010)
	Red deer		Liver	3/30 (10)	–	
	Roe deer		Liver	9/41 (21.9)	–	
Italy (Central)	Pig	Retail	Raw and dry liver sausages	10/45 (22.2) 1/23 (4.3)	3.44–5.34	Di Bartolo et al. (2015)
Italy (Central)	Wild boar	Hunting	Liver	55/164 (33.5)	–	Montagnaro et al. (2015)
Italy (Northern)	Wild boar	Hunting	Liver	12/320 (3.7)	–	Caruso et al. (2015a)
Italy (Northern)	Wild boar	Hunting	Liver	7/372 (1.9)	–	Serracca et al. (2015)
Italy	Pig	Slaughterhouse	Liver Lingual muscles	2/33 (6) 2/33 (6)	–	Di Bartolo et al. (2012)
Portugal	Wild boar	Hunting	Liver	20/80 (25)	–	Mesquita et al. (2016)
Spain	Pig	Slaughterhouse	Liver	1/39 (3)	–	Di Bartolo et al. (2012)
	Pig	Supermarket	Sausages	6/93 (6)	–	
Switzerland	Pig	Slaughterhouse	Liver	2/160 (1.3)	–	Müller et al. (2017)
Czech Republic	Wild boar	Hunting	Liver	50/438 (11.4)	7.27 (4.49–11.16)	Kubankova et al. (2015)
Czech Republic	Pig	Slaughterhouse	Liver Lingual muscles	2/40 (5) 1/40 (3)	–	Di Bartolo et al. (2012)
Netherlands	Wild boar	Hunting	Liver	2/102 (2)	–	Rutjes et al. (2010)
	Red deer		Liver Muscles	1/39 (3) 2/39 (5)	–	Rutjes et al. (2010)
Netherlands	Pig	Butcher	Liver	4/62 (6.5)	65 PCR-detectable units per g	Bouwknegt et al. (2007)
UK	Pig	Retail	Liver	1/76 (1.3)	6	Banks et al. (2010)
UK	Pig	Slaughterhouse	Liver	1/40 (2.5)	–	Berto et al. (2012)
		Butcher	Sausages	6/63 (9.5)	–	

HEV: hepatitis E virus; RNA: ribonucleic acid; RT-qPCR: reverse transcription real-time polymerase chain reaction.

*: Using ORF2- and ORF1-specific primers, respectively.

3.6.5. Blood products

Commercial blood products of animal origin, either from plasma or the cellular fraction of blood including whole blood, serve particular functions in different food products. Although they are mainly used in meat products, primarily to increase protein levels and enhance water binding and emulsifying capacity, advances in food technology mean that blood-derived products are beginning to be found as ingredients in non-meat processed food and dietary supplements (Hsieh and Ofori, 2011). However, the predominant role of plasma products in the meat industry is as a binder because of their ability to form gels upon heating. For example, the plasma proteins fibrinogen and thrombin are selectively cryo-precipitated from plasma and used as a natural binder in whole muscle processing. Usage of fibrinogen products in the EU currently requires a country by country approval. In May 2010, the European Parliament voted to ban the use of fibrinogen as meat glue. The ban, however, never took effect as in accordance with Commission directive 2010/67/EU, fibrinogen is permitted for use as a food additive for reconstituting food. EU countries still think differently about the nature of enzymes like fibrinogen and therefore criteria for categorising enzymes in council regulation no. 1332/2008 on food enzymes are currently the subject of discussion.

HEV inactivation/removal from blood products, for example, fibrinogen, may be influenced by processing conditions, such as heat treatment and stabilisers used (Farcet et al., 2016). Detection of HEV RNA in blood products has been reported; therefore processing conditions are critical to prevent HEV survival and contaminations of food products with HEV.

Blood is also used to produce sausages (e.g. morcilla (Spain), boudin noir (France)), which are composed of raw pork blood and other ingredients (fats, meats, spices, etc.), but sold cooked. No studies on the occurrence of HEV were conducted on blood sausages.

3.6.6. Food of non-animal origin

Contaminated water can be a vehicle of HEV particles transmission to fruits and vegetables, particularly for leafy greens and berries (Kokkinos et al., 2016). In Canada, HEV RNA has been detected in 1 out of 60 strawberry samples, 16 of which were found positive for human NoV and 2 for human rotaviruses. The HEV strain identified showed a high sequence identity with a HEV-3 strain detected in pigs on a farm in the same region (Brassard et al., 2012). In two studies conducted in European countries, 1 out of 38 (2.6%) frozen raspberries (Maunula et al., 2013) and 5 out of 146 (3.42%) fresh lettuce were positive for HEV RNA (Kokkinos et al., 2012). Molecular analysis of the virus strains was not achieved in either of the studies. The authors concluded that this resulted from an insufficient amount of viral RNA, suggesting a low level of HEV contamination, although the sensitivity of detection may also have been low. HEV RNA has also been detected in (2/230, 0.9%) herbs and spices (Loisy-Hamon and Leturnier, 2015). Despite some evidence of contamination of foods of non-animal origin by HEV, vegetarianism has been identified as a protective factor in several studies (Cossaboom et al., 2016; Sivasangeetha et al., 2016; Tedder et al., 2016).

Concluding remarks

- Pork meat is the major type of meat produced in the EU-28. The wild boar population is growing however, compared to pork meat, the amount consumed is much smaller.
- HEV has been reported in food of animal origin both at the slaughterhouse and point of sale. Products containing raw liver have been frequently found to be HEV-RNA-positive.
- HEV infection may be mainly linked to consumption of raw or under-cooked virus-contaminated liver and processed meat products.
- Blood from viraemic animals could be a potential source of HEV infection if used in food products (e.g. blood sausages) that are insufficiently cooked.
- Other blood-derived products such as fibrinogen are increasingly used as ingredients in meat and also in non-meat processed foods and dietary supplements and may constitute a risk only if not properly heat-treated.
- The role of the environment (e.g. organic fertilisers or irrigation water) as source for HEV contamination of food of non-animal origin is unknown and should be evaluated.
- A small number of studies have reported a low prevalence of HEV in shellfish, soft fruit and vegetables.

3.7. Environmental sources of HEV

The millions of pigs raised annually in individual countries within Europe produce many hundred thousands of tons of HEV-contaminated pig manure, much of which ends up in the environment. Also, urban sewage has been shown to contain HEV, and may be either treated or untreated and is discharged into surface waters. These contaminated surface waters may contribute in several ways to contamination of food of non-animal origin that may become also contaminated when they are fertilised by contaminated manure or manure products. Fruit and vegetables may become contaminated when they have been irrigated with contaminated surface water or have been treated by pesticides applied in HEV-contaminated water. It is not certain whether HEV may become internalised within plants, like some other enteric viruses (DiCaprio et al., 2015). If the contaminated surface water is in a shellfish harvesting area, the virus bioaccumulates in the digestive tract of the bivalve molluscs, thereby concentrating HEV. Also drinking water contamination may occur in cases of insufficient treatment of contaminated source waters, and HEV infections have been associated with animal manure contamination of drinking or irrigation water (Yugo and Meng, 2013). HEV RNA has been detected in irrigation water, soft fruit and vegetables, and in shellfish, indicating that HEV transmission may occur through environmental contamination (Kokkinos et al., 2012; Maunula et al., 2013).

3.7.1. Presence of HEV in urban sewage, manure and biosolids

Several studies have reported the detection of HEV RNA in sewage effluent, indicating the possibility of contamination of aquatic environments. Raw sewage has been shown to contain HEV strains closely related to those circulating in humans and animals (Yugo and Meng, 2013). Smith et al. (2016) summarised the frequent detection of HEV RNA in reported studies. In sewage from France (1 out of 4; 25%), Switzerland (40/124; 32%), India (80/144; 56%) and Spain (32–43%), relatively high percentages of HEV RNA-positive urban sewage samples have been described. A reported prevalence of 93% (14/15) of HEV RNA in untreated sewage samples from Edinburgh, Scotland, UK has been published (Smith et al., 2016). Lower figures have been described in other studies. In Italy, HEV RNA was detected in 1 out of 21 raw sewage samples but in none of 21 treated sewage samples (Iaconelli et al., 2015). In Japan, in 1 out of 99 tested raw sewage samples HEV-3 RNA was detected, and in none of 53 treated sewage samples (Ishida et al., 2012). In Italy, a molecular HEV screening of raw sewage samples from 11 wastewater treatment plants collected between 2008 and 2009 yielded 19 positives (16%), of which 18 belonged to HEV-1 and one to HEV-3. Contaminated sewage plants were evenly distributed throughout the country (La Rosa et al., 2010). HEV was detected in 13.5% of the wastewater samples collected in the northeast of Spain, when analysed by nested RT-PCR (nPCR). HEV RNA was detected in 9 of the 91 samples collected from raw sewage (5/37) and secondary treatment effluent (4/32; Rusinol et al., 2015).

Seasonal variations in the frequency of detection have been described in Switzerland, with 14% in the winter and 50% in the summer (Masclaux et al., 2013). This may relate to a concentration effect when water is scarcer in summer, but the effect of heat stress on hosts that may shed HEV in higher numbers remains to be elucidated. In some other countries, peaks of HEV infection have corresponded with periods of heavy rain and flooding, which may overwhelm the capacity of sewage treatment systems (Zhuang et al., 1991). Infectious particles have been reported to occur in raw sewage samples collected at a wastewater treatment plant in Barcelona (Spain). One of the 37 tested samples showed a positive result for HEV, which was typed as HEV-1. Infectivity was demonstrated by inoculation into rhesus monkeys (Pina et al., 1998).

A few Spanish studies demonstrated the presence of HEV RNA in sewage sludge and biosolids. HEV strains belonging to HEV-3 were frequently detected in low concentrations in urban sewage and biosolids and in slurry containing swine faeces (Albinana-Gimenez et al., 2006), but not in the river water samples studied. Clemente-Casares et al. (2009) detected HEV RNA in six sewage and two biosolid samples collected in urban wastewater treatment plants and in farm slurry samples in the Barcelona region, Spain. Typing revealed that strains were mainly HEV-3 and sporadically HEV-1 in urban sewage and biosolids, showing the simultaneous circulation of different HEV strains in the human population of the studied area. HEV-3 was identified in slaughterhouse sludge samples.

3.7.2. HEV contamination of surface waters

Discharge of wastewater, manure application to land and grazing of infected animals may contribute to HEV contamination of surface waters and sediment, and HEV RNA has been detected in surface waters in several countries: HEV-3 RNA was detected in 2 out of 12 river water samples in the Netherlands (Rutjes et al., 2009), in 1 out of 37 seawater samples in Japan (Ishida et al., 2012), in 1 out of 27 river samples in Italy (Iaconelli et al., 2015). In Slovenia, 60 surface waters were sampled throughout the country, of which two (3.3%) were positive for HEV RNA, one of them in the near vicinity of a pig farm (Steyer et al., 2011). Similar results (2/60 samples) were obtained from 60 surface water samples in Serbia (Lazic et al., 2015). A study conducted in the USA examined the presence of HEV RNA in watercourses adjacent to fields used for spreading large quantities of pig slurry. Surface water samples (n = 154) were collected from public access waters in proximity to these spray fields for 6 months, and were tested for HEV. HEV RNA was detected in one sample (Gentry-Shields et al., 2015). Kasorndorkbua et al. (2004) did not detect HEV in 28 surface water samples taken near pig farms in the USA during late summer and autumn. Drinking water contamination may occur in cases of insufficient treatment of contaminated source waters.

Givens et al. (2016) studied the presence of HEV RNA in relation to periods of pig manure application in a river basin area with extensive pig production. HEV RNA was detected in 45% of samples (n = 20) collected from the sampled river basin. This virus was not detected in the two samples collected from a control site. Within the river basin, 25% of the main-stem river samples and 58% of tributary were positive for HEV RNA. Prior to manure application (August 2011 and March 2012), HEV detection rates were similar between the main-stem (25%) and tributary (17%) samples.

After manure application (November 2011 and April 2012), HEV detections were significantly different ($p = 0.01$) between main-stem (25%) and tributary (100%) samples. The occurrence and concentration of HEV in surface waters increased significantly ($p = 0.04$) following manure application, with HEV being detected in 20% of samples prior to manure application and in 80% of samples after manure application. The increase in HEV RNA detected after manure application suggests that runoff may be an important transport mechanism of HEV to local surface waters.

Cases of HEV infection in Cornwall, UK are associated with coastal residence. The reason for this observation is unclear, but might be related to recreational exposure to beach areas exposed to HEV-contaminated 'run-off' from pig farms or sewage discharge (Hunter et al., 2016).

3.7.3. Stability of HEV in the environment

There are significant knowledge gaps on the survival of HEV in foods and the environment and the effect of decontamination procedures used in primary production food chain processes. The lack of a reliable infectivity assay has hindered such studies (Arthur and Gibson, 2015), and it is recommended that research be undertaken to develop an efficient HEV propagation system to facilitate the acquisition of important data on the survival of HEV in food and the environment, and its response to disinfection and decontamination procedures (Cook and Van der Poel, 2015).

This lack of information has also hindered the identification and validation of cultivable viruses that may be suitable as model or surrogate viruses for survival of HEV.

Temperature is considered to be the major factor determining virus inactivation in the environment. The stability of HEV-3 under different time-temperature combinations was assessed by Johne et al. (2016), who demonstrated that infectious HEV was detected up to 21 days at 37°C, up to 28 days at room temperature, and until the end of the experiment (56 days), with a 2.7-log decrease of infectious virus, at 4°C. Based on these data, a predictive model was established, which may help estimate HEV stability in the environment or food in the future (Johne et al., 2016).

To be able to predict viral stability in the environment, Bertrand et al. (2012) performed a statistical analysis of literature data on virus inactivation to develop an empirical formula for predicting inactivation of specific viruses in specific matrices as a function of temperature. Linear model fitting was applied to analyse the effects of temperature, virus species, detection method (cell culture or molecular methods), matrix (simple or complex) and temperature category ($< 50^{\circ}\text{C}$ and $\geq 50^{\circ}\text{C}$). As expected, virus inactivation was found to be faster at temperatures $\geq 50^{\circ}\text{C}$ than at temperatures $< 50^{\circ}\text{C}$, and there was also a significant temperature-matrix effect. Virus inactivation appeared to occur faster in complex (amongst others surface water, seawater, sewage and soil) than in simple matrices (synthetic media, drinking water and groundwater). HAV and poliovirus appeared to be the most resistant at temperatures $< 50^{\circ}\text{C}$ in simple matrices, with the time to first log reduction (TFL) ranging from 1.8 to 2.0 (0.63–3.1) days. No HEV data were included in this literature review. However, the number of days estimated to be required for the first log reduction of HEV in synthetic media at moderate temperatures (4°C, room temperature and 37°C; Johne et al., 2016) may be comparable to the modelled inactivation rate.

Concluding remarks

- Contamination of the environment with HEV from human and animal faecal waste may lead to contamination of drinking water, fruit and vegetables, and bivalve molluscs. HEV-3 infectivity was detected up to 28 days at room temperature in separated cell culture media suggesting that the virus could persist for several weeks in the environment. However, there are significant knowledge gaps on the survival of HEV and the effect of decontamination procedures used in primary production and food chain processes. The lack of a reliable infectivity assay or surrogate model has hindered such studies.

3.8. Possible control measures along the food chain and evaluation of decontamination treatments

In recent years, it has been shown that the majority of HE cases reported in Europe are of autochthonous origin. Several possibilities are considered as the source of the infection: foods originating from infected animals (domestic pigs and wild boar); food contaminated with the excreta of

infected animals; indirect transfer through contamination of the environment; and direct contact with HEV-infected animals.⁸ Although direct transmission from humans may also be a source of infection for humans, this is not included in the present opinion, but it is assumed that reduction of sources of infection will also have a beneficial effect reducing the opportunities for secondary transmission.

Food may be contaminated by HEV at various steps in the food chain from farm to processing plants and point of sale. Control methods for HEV in food will differ between commodities depending on the risk of contamination of the specific products. This chapter summarises existing preventive measures in place according to current legislation, possible control measures to improve the efficiency of existing control options and effects of treatments used in food processing, as well as identified data gaps.

3.8.1. Potential for control of HEV in the pork food chain

Food-borne transmission of HEV is mainly through consumption of products originating from reservoir animals, especially by consumption of raw or inadequately heat-treated offal and meat. According to a case-control study in Germany during 2006 and 2007 involving 45 cases of HE and 135 control subjects, consumption of offal (41% vs 19%; OR 2.7; 95% CI 1.2–6.2) was independently associated with autochthonous source of the infection. In contrast, direct animal contact appeared to not play a major role in clinical HEV infections (Wichmann et al., 2008).

The current control measures for food of animal origin rely on EU legislation; Regulations EC 853/2004 and 854/2004. To date, no specific legislation or specific measures for HEV exist. There are currently no official control policies regarding HEV in animals. Infected animals often do not show symptoms of infection; therefore, they can be sent for slaughter and contaminated organs and meat will enter the food supply chain. Control options focused on products of animal origin, and possible novel control options are identified in 'Scientific opinion on an update on the present knowledge on the occurrence and control of food-borne viruses' (EFSA BIOHAZ Panel, 2011a) and ACMSF (Brien et al., 2015).

3.8.1.1. Potential for control of HEV at farm level

The prevention of HEV introduction into pig breeding pyramids and production herds and thus the reduction of HEV-infected pigs at the time of slaughter could be of major benefit if there are sufficient HEV-free sources of pigs to establish infection-free networks. In those countries where national surveys have been carried out, the seroprevalence is very high, suggesting that most commercial-scale pig farms producing slaughter pigs are likely to be infected. It is also likely that nucleus and multiplier breeding herds will also be infected, but this remains to be investigated and more studies on prevalence and vertical and horizontal transmission of HEV within primary pig breeding herds are required, along with a quantitative risk assessment to evaluate the potential benefit to public health of reductions in prevalence or viraemia in pigs at slaughter.

The transmission of HEV virus between pigs is strongly influenced by environmental faecal contamination, which suggests the possibility to reduce the prevalence of infected pigs by appropriate farm management, hygiene (including effective disinfection of pig housing and equipment between batches) and biosecurity measures (Rose and Pavio, 2014).

One possible control option could be to prevent the introduction of HEV and thereby keep herds free from infection. If this is not possible, measures could be taken to reduce the number of infected animals by the time of slaughter to minimise the risk of viral transmission to humans.

The main principles for reducing the risk of transmission to humans of HEV with infected pig herds are likely to be similar to those used for control of other faecal-oral infections in terms of preventing introduction of infected animals, batch production, effective cleaning and disinfection between batches, control of wildlife reservoirs and vectors and managing pig flow to avoid initial exposure to infection late in the finishing stages, to allow viraemia, liver infection and shedding of virus to subside before slaughter and preventing contamination at slaughter.

HEV was detected in 30/88 Canadian pig farms (34.1% (95% CI 25.0%, 44.5%)). Farm-level prevalence varied with province and sampling intensity. Requiring shower-in and providing boots for visitors were significant predictors ($p < 0.05$) in single fixed-effect mixed logistic regression analysis for

⁸ http://www.bfr.bund.de/en/questions_and_answers_on_the_transmission_of_the_hepatitis_e_virus_through_wild_boars_and_domestic_pigs_and_foods_derived_from_them-196615.html

reduced detection of HEV. Such biosecurity measures and not obtaining feeder pigs from multiple sources were associated with reduced odds of detecting HEV (Wilhelm et al., 2016).

A risk factor analysis, based on a previous prevalence study of 185 farms, was conducted on 90 farms located in Western France. At least one HEV RNA-positive liver was found in 30% of the sampled farms, while seroprevalence in slaughter-age pigs at the farm level was almost 75%. The risk of having HEV-positive livers was increased by early slaughter, genetic background, lack of hygiene measures and a surface drinking water source. High HEV seroprevalence was associated with mixing practices at the nursery stage and hygiene conditions (Walachowski et al., 2014).

In Lao People's Democratic Republic, pigs are kept in close contact with families. A total of 59 villages, 895 humans and 647 pigs were sampled and serologically tested for zoonotic pathogens including HEV. Seroprevalence of zoonotic pathogens in humans was high for HEV (48.6%, –77.7% depending on region). Multiple correspondence analysis and hierarchical clustering of principal components was performed on descriptive data of human hygiene practices, contact with pigs and consumption of pork products. Three clusters were identified. Cluster 1 had low pig contact and good hygiene practices. Most people in cluster 2 were involved in pig slaughter (83.7%), handled raw meat or offal (99.4%), and consumed raw pigs' blood (76.4%). Compared with cluster 1, cluster 2 had an increased risk of testing seropositive for HEV. Cluster 3 had the lowest sanitation standards and the highest risk of HEV. Farmers who kept their pigs tethered (as opposed to penned) and disposed of manure in water sources had 0.85 (95% CI: 0.18–0.91) and 2.39 (95% CI: 1.07–5.34) times the odds of having pigs test seropositive for HEV, respectively, demonstrating that avoidance of faecal accumulation exposure is likely to reduce the risk of HEV infection for both pigs and people (Holt et al., 2016).

The impact of passive maternal immunity on HEV transmission in piglets was investigated using longitudinal serological data from seven pig farms in France and Spain. The efficiency of transmission was 13 times lower in piglets with maternally derived antibodies than in fully susceptible piglets. Median infection-free times, based on herd-specific estimates, ranged between 8.7 and 13.8 weeks in all but one herd, and this herd exhibited a different profile with a relatively low prevalence of infected pigs (50% at slaughter age) despite similar proportions of passively immune individuals after colostrum intake. These results suggest that the age at HEV infection is not only dependent upon the proportion of piglets with colostrum antibodies, but is also likely to be linked to farm-specific husbandry practices, for example, batching of piglets after weaning and farm hygiene standards (Andraud et al., 2014).

The presence of some anti-HEV antibody-free herds in intensive pig farming areas of Germany may indicate that it is feasible to establish and sustain HEV-free pig herds (Krumbholz et al., 2013). Similar findings were reported from Brazil, where HEV infection was less likely to be found in large-scale pig production (da Costa Lana et al., 2014), but more intensive sampling and test validation is required to confirm a truly relevant HEV-free status (Wang et al., 2015a) and larger scale government farms were more likely to be infected than smaller farms in Bhutan (Monger et al., 2017). The pig industry would have to be willing to establish such SPF herds, which has not proved to be economically feasible in most countries for other zoonotic pathogens associated with pigs, for example, *Salmonella*, in the absence of legislation or financial penalties.

Few data are available on how best to diagnose and eliminate HEV in pig herds used for medical purposes or the importance of viral infection at the stage of harvest. In one study, several groups of animals used for xenotransplantation were found to be RNA-positive in both serum and faeces for HEV genotype 3. Viraemia was found in animals up to 3.6 years old, which is much longer than originally thought. Segregation of animals at an early age and subsequent hygiene barriers prevented spread of infection, suggesting that weaning to clean accommodation could be a means of establishing HEV-free herds if suitable within-herd biosecurity can be maintained (Busby et al., 2013).

The mechanisms of pathogenesis and persistence of HEV in the host appear to be substantially immune-mediated, suggesting that widespread use of vaccination could be a valuable control measure (Krain et al., 2014). Porcine reproductive and respiratory syndrome virus (PRRSV) reduces the immune response in pigs. PRRSV is very common in pig production and is suspected to influence HEV infection dynamics. In an experimental HEV/PRRSV co-infection study in specific-pathogen-free (SPF) pigs, follow-up of co-infected animals showed that HEV shedding was delayed by a factor of 1.9 in coinfecting pigs compared with HEV-only infected pigs, and identification of the specific immune response was delayed by a factor of 1.6. HEV shedding was significantly increased by coinfection and substantially extended (48.6 vs 9.7 days for HEV only). The long-term HEV shedding was significantly correlated with the delayed humoral response in coinfecting pigs. The transmission rate was estimated to be 4.7 times higher in the case of co-infection than in HEV-only infected pigs (0.70 and 0.15 per

day, respectively). HEV infection susceptibility was increased by a factor of 3.3, showing the major impact of PRRSV infection on HEV dynamics. Control of PRRSV by management (e.g. by eradication or vaccination) is therefore likely to have a beneficial impact of HEV infection (Salines et al., 2015).

The HEV vaccine was licensed in China for the prevention of HEV infection and HEV-related diseases in humans with a high level of proven safety and efficacy, but so far studies of its use in pigs have not been published and such challenge studies would be desirable. Vaccination using 2 mcg doses of the vaccine provided good protection in laboratory rabbits against both rabbit HEV and human HEV-4 infection (Zhang et al., 2014, 2015). Sanford et al. (2012) expressed and characterised N-terminal truncated ORF2 capsid antigens derived from swine, rat and avian HEV strains, and evaluated their cross-protective ability in a pig challenge model. There was no detectable difference in the level or pattern of antibody responses among the vaccinated groups. However, according to detection of HEV in faeces of pigs challenged with HEV-3 at 4 weeks post-vaccination, only the avian HEV and swine HEV capsid antigens induced certain levels of protection against the HEV-3. A trivalent subunit vaccine for HEV, NoV and astrovirus was developed by fusion of the dimeric P domains of the three viruses. This vaccine elicited significantly higher antibody responses in mice against all three P domains than those produced by a vaccine comprising a mixture of the three free P domains. Furthermore, the post-immune antisera of the trivalent vaccine showed significantly higher neutralising titres against HEV infection in cell culture than the post-immune sera of the mixed vaccine (Xia et al., 2016). Multivalent live vector vaccines, incorporating antigens of clinical relevance to pigs, may therefore offer a cost-effective method of enhancing uptake of preventive vaccination in the pig industry (Trabelsi et al., 2014; Wei et al., 2014), and may also reduce vertical transmission (Wang et al., 2015a; Abraham, 2016). Modelling studies have suggested a beneficial effect of vaccination in pig herds, even if total protection cannot be achieved (Backer et al., 2012). There is a need for both controlled challenge studies and field-based intervention studies to evaluate the potential impact of vaccination of pig herds against HEV. A regulatory and commercial climate that encourages research and development of multivalent live recombinant vaccines that combine antigens relevant to animal health and welfare and zoonotic infections, including their safety and efficacy, is recommended to help stimulate protective vaccination programmes for pathogens of public health relevance in food animal production.

Transcutaneous immunisation using nanoparticle-adjuvanted vaccines has been proposed as a method for increasing the immunogenicity and ease of application for HEV vaccines (Kaurav et al., 2016).

Despite considerable research in recent years, the level of awareness of HEV risk associated with pig meat products and other reservoirs and sources is low, so improved dissemination of information and advice to consumers and those working with potential sources of infection is recommended (Alkali et al., 2015).

3.8.1.2. Potential for control at slaughterhouses, meat processing plants and retail

Mandatory ante-mortem inspection of individual animals and post-mortem inspection and sampling of individual carcasses are required to help prevent certain meat-borne zoonoses, such as parasitic (e.g. trichinellosis) and bacterial infections (e.g. bovine tuberculosis). Animals harbouring microbiological pathogens such as HEV cannot be recognised during routine meat inspections. Present measures to avoid or to reduce faecal contamination of carcasses have an impact on possible surface contamination of carcasses with HEV originating from faeces (EFSA BIOHAZ Panel, 2011a). Compliance with good practice during transport, lairage and at the slaughterhouse as well as during processing and storage should reduce the risk of HEV cross-contamination of pork meat, for example, contamination of pig skin and pharynx with faeces, which may occur during movement of the animals before slaughter or involves carcasses during stunning or subsequent handling. If skinning is applied without hot steaming or burning, contaminating viruses could be transferred to the surface of meat and increase the risk of cross-contamination. The efficacy of the scald tank (temperature above 60°C for several minutes or efficient steam scalding) and singeing (including double singeing) is likely to be important for carcasses that are not skinned. Procedures such as bunging and hot water washes may also be applied to minimise faecal contamination, and blast freezing is increasingly common and may have some antimicrobial effect, but possibly less so against viruses than bacteria. Pork meat and fat, equipment and utensils could also be contaminated especially in the case of inappropriate removal or accidental perforation of the intestine.

Because high viral load can be present in faeces, bile and liver of infected animals (Table 2), liver, gall bladder and other internal organs should be kept separate from the rest of the carcass. Special care should be taken to prevent direct contact of meat (muscle) and fat with faeces or bile.

Dedicated equipment and utensils, particularly knives, should be used only for their specific operations. This rule is imperative when removing internal organs. Knives and cutting tools in robots should be treated with hot water (85°C for 15 s) after each operation.

As slaughterhouse workers show higher anti-HEV prevalence compared with people without occupational exposure to pigs (42% v 16%; Krumbholz et al., 2012), abattoir staff and food handlers should be educated about HEV and follow good hygiene practices, and special attention should be paid to abrasions on the skin. The risk of infection can be reduced by wearing the prescribed protective clothing. Cross-contamination of other food during handling and preparation is likely a route of HEV transmission (Wichmann et al., 2008), therefore procedures leading to cross-contamination should be avoided.

Infectious viral particles can be present in blood and liver of reservoir animals and thus within meat or offal at the time of slaughtering, therefore measures involving improvements in hygiene will have a lower impact on HEV transmission via meat and offal and HEV should be inactivated during subsequent processing (Brien et al., 2015). Therefore, testing of meat and offal to be eaten raw or lightly cooked, in which HEV will not be inactivated during subsequent processing, should be considered.

In particular, provision of information to vulnerable groups (e.g. persons with a weakened immune system, pre-existing liver injury) may help prevent the most serious HEV infections.

3.8.2. Potential for control of hunted game

Several studies confirm that after domestic pigs, wild boar and likely deer may also play a role as a reservoir of HEV. In a case-control study performed by Wichmann et al. (2008; 45 case subjects and 135 control subjects), consumption of wild boar meat (20% vs 7%; OR, 4.3; 95% CI, 1.2–15.9) was associated with autochthonous HEV infection. In contrast, consumption of wild-animal meat other than wild boar (18% vs 23.0%; OR 0.7; 95% CI 0.29–1.73) does not appear to be an important source of HEV infection in European countries. Although direct animal contact seems to play no major role of HEV transmission (Wichmann et al., 2008), during skinning and disembowelling of HEV animal reservoirs like wild boars, hunters may have direct contact with blood or other body fluids if they do not wear any barrier protection, such as protective gloves (Schielke et al., 2015).

No specific microbiological criteria for game meat exist as yet in the EU legislation. Regulation (EC) no 2073/2005, which states values for total viable count (TVC) and Enterobacteriaceae in the carcasses of pigs and ruminants, can be used for game meat. The microbiological quality of wild boar meat is generally considered to be similar to that of domestic pigs (Borilova et al., 2016).

General recommendations to reduce the risk to hunters and others handling carcasses of wild game posed by HEV-infected animals are mainly characterised below.

- Hunters who used protective gloves on a regular basis had an 88% lower anti-HEV prevalence as compared with hunters disembowelling wild boars in the same area but wearing gloves never, seldom or sometimes (age-adjusted PR 0.12; 95% CI 0.02–0.86; Schielke et al., 2015). Therefore, wearing protective gloves during disembowelling and paying attention to abrasions on the skin when handling hunted game should be recommended.
- The knives for disembowelling, knives and other utensils (e.g. chopping boards) for raw venison and offal should be used only for these purposes to avoid cross-contamination. Hands should be properly washed after disembowelling and handling of wild boar, venison or their offal.
- Hunters and others handling carcasses as well as the general public should be educated about HEV transmission associated with game mammals.
- Sufficient heat-treatment of wild boar meat, venison and offal prior to consumption should be performed (see Section 3.8.5).

3.8.3. Potential for control of bivalve molluscs' food chain

General hygiene requirements regarding bivalve mollusc food chains are laid down in Regulations (EC) no 852/2004 and (EC) no 854/2004 and their amendments. Specific requirements for the hygiene of live molluscs are covered by Regulation (EC) no 853/2004 and Regulation (EC) no 2073/2005 (EFSA

BIOHAZ Panel, 2015). Risk management legislation for sanitary production of bivalve molluscs worldwide depends on the impact of human faecal pollution and prescription of food processing measures prior to placing the molluscs on the market. Legislative standards controlling permitted levels of faecal pollution are based on indicator bacteria, *E. coli* or faecal coliforms. Within the EU, all commercial production areas must be monitored (on a periodic basis) for *E. coli* in molluscs' flesh, which determines the classification of production areas: A (cleanest), B or C (most contaminated; EFSA BIOHAZ Panel, 2015). The method may also be used for determining compliance with the end-product standards. While class A molluscs may be placed directly on the market for human consumption, class B or Class C molluscs require relaying in natural beds, or depuration in commercial tanks, or thermal processing at defined time and temperature conditions prior to placing the molluscs on the market. Molluscs that do not conform to any of the classification categories are not allowed to be placed on the market for human consumption (EFSA BIOHAZ Panel, 2015).

Recommendations to improve efficiency of the control options in existing EU legislation are elaborated, for example, in EFSA Scientific Opinions (EFSA BIOHAZ Panel, 2011a, 2015) and ACMSF (Brien et al., 2015). These recommendations are mainly related to NoV and HAV. They could be used for HEV with suitable modifications as described below.

Once viral contamination of bivalve molluscs has occurred, removal or inactivation of the virus by processes that retain the sensory characteristics of the molluscs may be difficult. The main measures should be to prevent viral contamination of the molluscs by improving environmental conditions (particularly water quality) in production and harvesting areas and tightening of indicator standards reflecting the presence of human and animal faecal pollution for harvest areas (Brien et al., 2015). Alternative indicators such as bacteriophages, polyomaviruses or adenoviruses have been suggested (Dore et al., 2000; Formiga-Cruz et al., 2003; Rusinol et al., 2014). Because no significant correlation between the presence of human adenovirus and NoV, HAV and HEV has been reported (Diez-Valcarce et al., 2012), other indicators (e.g. porcine adenovirus, polyomavirus) and direct monitoring of HEV in water should be considered. However, more data are required before the suitability of an enteric viral indicator can be validated.

Epidemiological and laboratory studies show that depuration times and conditions currently used are inadequate (Lees, 2000; Richards et al., 2010) due to the slower removal of viral contamination. Molluscs compliant with the *E. coli* standard may still contain enteric viruses and thus cause outbreaks (Dore et al., 2010; Maalouf et al., 2010). Depuration and relaying may be improved by optimising process parameters to enhance virus reduction (e.g. depuration times, water temperature). However, no data on impact of depuration and relaying on HEV presence in molluscs are currently available (Brien et al., 2015).

3.8.4. Potential for control of fruit and vegetable food chains

Primary production, as well as processing plants, needs to fulfil the general rules of hygiene laid down in Regulation EC no 852/2004. So far, neither HEV nor other food-borne viruses are included in EU legislation.

Because limited information is available about the stability of HEV in the environment outside the host organism as well as its resistance to decontamination procedures, general recommendations regarding the other food-borne viruses should be followed. Recommendations on fresh produce (fruit and vegetables) are well described by the EFSA Scientific Opinions (EFSA BIOHAZ Panel, 2013, 2014), and by *Codex Alimentarius* 'Guidelines on the application of general principles of food hygiene to the control of viruses in food' (CAC, 2012). The BIOHAZ Panel (EFSA BIOHAZ Panel, 2013, 2014) concluded that: appropriate implementation of food safety management systems including Good Agricultural Practices (GAP), Good Hygiene Practices (GHP) and Good Manufacturing Practices (GMP) should be the primary objective of operators producing fruits and vegetables. Attention should be paid to the selection of the water sources for irrigation, agricultural chemicals (e.g. pesticides, fungicides and fertilisers), and in particular to the avoidance of the use or the ingress of water contaminated by human sewage as well as agricultural effluents and flooding (EFSA BIOHAZ Panel, 2012, 2014a,b).

Leafy greens eaten raw as salads do not include any processing steps or control points that will ensure removal or inactivation of viral contamination; therefore, it is particularly important to consider risk factors (and consequentially mitigation options) at the point of production. Some measures to reduce the risk of contamination of fresh produce during primary production are implemented and they could be used for HEV reduction with suitable modifications as described below.

Fertilisation can be done with chemical and/or organic fertilisers. Organic fertilisers, such as animal manure, may introduce faecal pathogenic bacteria, viruses and parasites to fruits and vegetables if manure is not adequately aged or otherwise treated before application (Mawdsley et al., 1995; Strawn et al., 2013). Therefore, only properly treated organic fertilisers should be used. Manure piles and outdoor pig units located next to growing operations may represent a risk of contamination via run-off, vertebrate and insect vectors, dust or aerosols (Suslow et al., 2003; Brandl, 2006; James, 2006).

In Europe, the main water sources are surface waters (rivers, lakes) and reservoirs supplied by well water or rain water (EFSA BIOHAZ Panel, 2014). Water that has been contaminated with viruses, for example, from a nearby sewage outflow, and is then used for irrigation or pesticide application during primary production can cause contamination of fresh produce (EFSA BIOHAZ Panel, 2011a, 2013, 2014; DiCaprio et al., 2017). Therefore, according to Codex Alimentarius recommendations clean water should be used for spray irrigation or application of chemical fertilisers or pesticides. Because *E. coli* was identified as a suitable indicator of faecal contamination for a Hygiene Criterion at primary production of fruit and vegetables (EFSA BIOHAZ Panel, 2013, 2014), new evidence suggests that alternative indicators such as bacteriophages, polyomaviruses or adenoviruses and direct testing of HEV in water should be considered. However, more data are required before the suitability of an enteric viral indicator can be validated. In FAO/WHO (2008), it was agreed that subsurface irrigation lowers the risk of pathogen transfer from water to growing plants.

Where higher risk water sources are used for irrigation (e.g. surface waters), delivery systems that prevent the water coming into contact with the edible portion of the plant, such as low-volume sprays, drip, trickle tape, furrow or underground irrigation should be used to reduce the risk of contamination.

Access of HEV animal reservoirs (domestic or wildlife) should be restricted. However, while domestic animals may be separated from growing areas, it can be more difficult to control access of wild animals.

There is the possibility for virus contamination from various food products to spread via cross-contamination through contact with food processing or preparation surfaces. For example, this could occur through cutting of a contaminated item followed by using the same utensil to cut uncontaminated items without adequately cleaning them first (Escudero et al., 2012; Wang et al., 2013a). Therefore, harvesting equipment, for example, crates, baskets, should be kept clean. All equipment and surfaces, such as containers or conveyor belts, which come into contact with food should be cleaned with clean water according to Codex Alimentarius principles water and disinfected after use.

Food handlers should be educated and implement GHP and avoid cross-contamination although for HEV human cases resulting from contamination of food by handlers has not so far been demonstrated.

Clean water according to Codex Alimentarius principles should be used as a minimum for post-harvest washing stages, whereas potable water should be used for the final washes and for cooling or freezing. The Codex Committee on Food Hygiene guidelines for control of virus contamination of food (CAC, 2012) recommend that efforts should be made to use only clean or potable water during production and processing.

Disinfection of fruits and vegetables is performed by some producers/processors; however, disinfection procedures used in the food industry may only have limited effect on HEV (see Section 3.8.5).

Concluding remarks

- Present control measures in the pork production chain are based on prevention of certain meat-borne parasitic or bacterial zoonoses and are not sufficient to prevent presence of HEV in pork.
- Regulations related to hygienic measures for foods of animal origin and control of products of animal origin for consumption are laid down in EU legislations 853/2004 and 854/2004. Ante-mortem and post-mortem inspection are not able to detect HEV, which may be present in liver or meat at the time of slaughter.
- For the reduction of the HEV transmission in pig farms, some management-based methods such as batching that can influence the time of first exposure to HEV and farm decontamination measures for potential reduction of HEV risk in pig herds have been described, but need to be confirmed in well-designed intervention studies.
- Vaccination of pigs is a potential control option, as protection of rabbits has been demonstrated by the use of a human vaccine, but the efficiency of vaccination of pigs to

prevent human disease requires further investigation. No vaccine is currently commercially available.

- Evaluation of the potential impact of vaccination of pig herds against HEV and other options such as HEV-free pig herds and the impact of management methods should be carried out.
- Control options mentioned in EFSA Scientific Opinions for NoV and HAV could also be beneficial for HEV.

3.8.5. Effects of treatments used in food processing

3.8.5.1. Effect of time–temperature combinations on HEV

Thermal treatment to reduce virus load is a common strategy in food industries. Few papers are available on the stability of HEV, evaluated by measuring the infectivity of the virus for cells, by capsid integrity assays using RNase treatment followed by RNA detection (measured as RNA-protected genome equivalent (RGE)) or by *in vivo* inoculation in animals. The methods are not comparable, for example, RNA detection results in an overestimation of the virus infectivity as RNA can be detected from damaged viral particles (Johns et al., 2016). However, results obtained can provide an estimation of virus resistance to food control treatments if such treatments would be expected to degrade RNA.

Data on both long- and short-term storage of HEV were obtained by treatments of HEV at various combinations of temperature and time. Studies reported a rapid initial decline of genome equivalent (GE or RGE) or focus forming unit (FFU) followed by a slower inactivation/reduction step. Besides the evidence that an RNase-protected genome degrades much more slowly than the reduction in infectivity, the studies showed that HEV is stable under long-term storage conditions and remains infectious for several weeks at room temperature (Schielke et al., 2011; Johns et al., 2016).

Table 4: Effect of time–temperature combinations on HEV

Genotype	Cell culture	Initial inoculum (titre)	Test for measure infectivity	Temperature/ time	Measure of infectivity	Reference
HEV-3	–	Homogenates of infected wild boar liver (3×10^6 RNase protected GE)	Monitoring of viral RNA*	4°C 1 h 22°C 3 days 37°C 7 days 56°C 15 min 56°C 30–60 min 60°C 60 min 95°C 1 min	0.34 log reduction 0.45 log reduction 1.24 log reduction < 1 log reduction 3–4 log reduction 3–4 log reduction 3–4 log reduction	Schielke et al. (2011)
HEV-3	PCL/PRF/5	Virus suspension in PBS (2×10^6 GE)	Monitoring of viral RNA	25°C 30 min 56°C 30 min 70°C 10 min 95°C 1 min	From day 16** From day 20 No RNA No RNA	Tanaka et al. (2007)
HEV-3; HEV-4	A549	Virus suspension in PBS (3.2–5.8/mL non-detectable end-point log dilution)	Monitoring of viral RNA	60°C 30 min	No RNA	Yunoki et al. (2008)
		Virus suspension in 25% human serum albumin	Monitoring of viral RNA	60°C 30 min	1–2.2 log reduction	
HEV-3	PCL/PRF/5 3D system	Virus suspension in PBS	Monitoring of viral RNA	56°C 1 h 100°C 15 min	From day 62 No RNA	Cook and Van der Poel (2015)

Genotype	Cell culture	Initial inoculum (titre)	Test for measure infectivity	Temperature/ time	Measure of infectivity	Reference
HEV-3	A549/D3	Virus from supernatant of infected cells*** (10 ⁴ FFU)	Monitoring of FFU	50°C 1 min 60°C 1 min 65°C 1 min 70°C 1 min 70°C 2 min 75°C 1 min 80°C 1 min	< 0.5 log reduction < 1.3 log reduction < 2.6 log reduction < 2.9 log reduction No detection (> 3.9 log reduction) < 3.4 log reduction No detection (> 3.5 log reduction)	Johne et al. (2016)
HEV-3	HepaRG or PICM-19	Filtered pig faecal suspension (4 × 10 ⁵ GE)	Monitoring of viral RNA	56°C for 60 min 95°C for 5 min	No detection	Rogée et al. (2013)

HEV: hepatitis E virus; RNA: ribonucleic acid; GE: genome equivalent; PBS: phosphate-buffered saline; FFU: focus forming unit.

*: Capsid integrity assays using RNase treatment followed by RNA detection.

**: RNA first detection in cell culture from day 16 post-infection.

***: Virus was recovered from the supernatant (growth medium without FCS) of infected cells.

The results reported in Table 4 show that HEV virus was affected by heat treatment delaying the time of detectable viral RNA on inoculated cells (e.g. detectable RNA from day 20 after treatment at 56°C for 30 min; Tanaka et al., 2007) or reducing the number of FFU detected (< 2.6 log reduction after treatment at 65°C for 1 min; Johne et al., 2016), but the virus was not totally inactivated (Cook and Van der Poel, 2015). Total inactivation was observed at higher temperatures, for example, higher than > 56°C for 1 h (Rogée et al., 2013), or a shorter time (1–2 min) but at higher temperature (Tanaka et al., 2007; Yunoki et al., 2008; Johne et al., 2016). Results are not comparable, different combinations of time and temperatures were used and the residual infectivity was measured by monitoring the presence of viral RNA or by FFU (Table 4). However, the results obtained provide an indication of HEV thermal resistance at least in the conditions tested.

Heat treatment at 60°C for 30 min in albumin was less effective compared with total HEV inactivation observed in control experiments using buffer rather than albumin (Tanaka et al., 2007; Yunoki et al., 2008). A heat treatment of 180 min at 58°C inactivated the virus in albumin (mean reduction of 3.1 log 50% Tissue culture Infective Dose (TCID₅₀)), while the inactivation was more rapid after 60 min (mean reduction of 3.5 log TCID₅₀), using virus stock in a buffer solution (Farcet et al., 2016).

All viruses are more resistant to heat treatment when embedded within tissues or other food matrices that afford protection against the effect of heat or other noxious agents that occurs when individual pathogens are present in suspension, and thereby exposed over their whole surface (Arthur and Gibson, 2015; Bozkurt et al., 2015; Emmoth et al., 2016), so HEV resistance to heat treatments can also vary depending on the matrix (e.g. faeces, liver, percentage of fat). Virus embedded in a slab of meat is expected to require a higher temperature to be inactivated (Emerson et al., 2005) than that determined in several cell-culture experiments reported (Table 4).

The thermal stability of HEV was analysed by using faecal suspensions of genotypes 1 and 2. The most resistant strain, HEV-1, showed a reduction of 95% of infectivity in cultured cells (HepG2/C3A) after treatment at 56°C for 15 min, a 1% residual infectivity after 60 min at the same temperature, and no residual infectivity at 66°C for 1 h (Emerson et al., 2005). These data indicate that the susceptibility of HEV to heat differed between HEV strains, where some were inactivated nearly completely when maintained at 56°C for 1 h, whereas for others 20% remained infective after being maintained at 60°C. A preliminary analysis of the decay of HEV genotype 1 (Akluj strain) based on these data suggests that a thermostable fraction of HEV may exist (Figure 5). This residual fraction could be the main determinant for the public health risk relating to lightly cooked products (Bouwknegt et al., 2013). Besides HEV strain characteristics, also the origin of the virus, serum- or faeces derived HEV may determine heat sensitivity. Yunoki et al. (2016) described that human serum-derived HEV showed some heat resistance during the first phase at heat inactivation. Detergent treated human serum-derived HEV demonstrated a similar heat-resistant property as that of swine faeces-derived HEV. These results suggested that the lipid associated with the viral particles may affect the heat sensitivity of HEV.

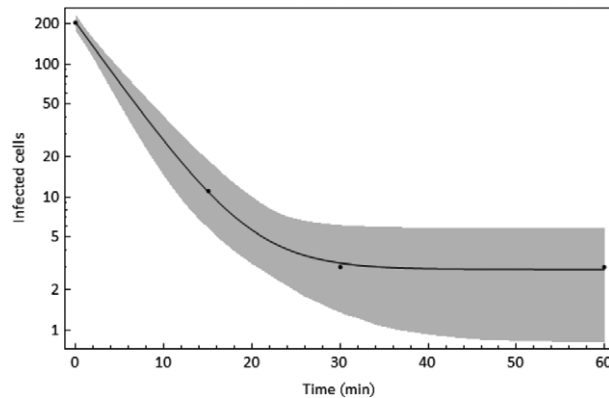


Figure 5: Inactivation curve for HEV, based on data from Emerson et al. (2005). These experiments showed, for HEV-1 strain, that the initial infectivity decay is large, but is minimal during the second phase (Bouwknegt et al., 2013)

Feagins et al. (2008) assessed the infectivity of cooked liver positive for HEV-3 by intravenous inoculation of the treated sample homogenates (GE titre unknown) into healthy pigs. Both liver blocks, boiled for 5 min or stir-fried (internal temperature 71°C), showed no residual infectivity in pigs. However, incubation at 56°C for 1 h did not inactivate the virus (Feagins et al., 2008). In contrast, pâté-like preparations (30% HEV-3-infected liver 2.24×10^7 GE/g; 48% fat) treated at different time/temperature combinations, ranging between 62°C and 71°C and 5–20 min, showed residual infectivity when experimentally inoculated into a group of pigs. Only treatments at 71°C for 20 min resulted in total loss of infectivity (Barnaud et al., 2012). Residual infectious virus was still observed when the pâté was heated at 62°C for 120 min, at 68°C for up to 20 min, and 71°C up to 10 min (Barnaud et al., 2012).

Data on HEV inactivation in bivalves are still not available, heat processing can be very effective if performed correctly. A HAV thermal inactivation model was used to identify equivalent (achieving the same log reduction) time–temperature combinations to 90°C for 90 s without considering the effect of heat-up and cool-down times on virus inactivation, between 72°C and 100°C such as 72°C for 407 s, 76°C for 291 s, etc. Evaluation against inactivation of HAV in whole bivalve molluscs showed that the observed HAV inactivation is in general higher than predicted (EFSA BIOHAZ Panel, 2015).

There are no epidemiological data (no reported HAV or NoV) to suggest that the current approved heat treatment criteria in EU legislation (a minimum of 90°C for 90 s) are not sufficiently protective for public health (EFSA BIOHAZ Panel, 2015). This has proved to be effective for both HAV and NoV (Appleton, 2000). As HAV appears to be more robust and requires more thorough cooking for effective inactivation than HEV (Emerson et al., 2005), the controls are likely to be effective. Conditions for heat treatment should be validated for the specific ability to inactivate HEV under commercial conditions.

3.8.5.2. UV

Ultraviolet (UV) light is a non-ionising radiation with germicidal properties at wavelengths in the range of 200–280 nm. It can inactivate food-borne microorganisms by means of nucleic acid damage without substantially heating the food, producing safer food and preserving high sensory and nutritional values. UV radiation is, however, only suitable for decontamination of surfaces or thin layers of clear liquids (Lado and Yousef, 2002).

Ultraviolet treatments of human HEV-3 viral suspension using low UV fluence ranging between 195 and 269 J/m² result in a 99.99% reduction of infectivity (reduction up to 4 log₁₀), as demonstrated by measuring both viral RNA and *in vitro* infectivity. The UV dose level required to obtain a consistent inactivation is below that required by international guidelines for water disinfection (Guerrero-Latorre et al., 2016).

In a study conducted by Berto et al. (as cited in FSA, 2014), a homogenate of HEV-3-infected pig liver was exposed for 20, 30 and 50 min to UV light by a lamp producing 30 W UVC and then inoculated into PCL/PRF/5 cultivated by 3D system. The effect of UV light was monitored for evidence of viral replication by detection of viral RNA. The viral RNA was detectable in the cell culture 60 days post-inoculation. The results indicate that UV light has only limited effects against HEV, as residual infectivity was observed. Because a HEV-positive liver homogenate was used, the virus was embedded

in the liver cells resulting in less exposure to UV irradiation. Data available so far are not sufficient to clearly state the effect of UV treatments on HEV, and further studies are needed.

3.8.5.3. Chlorine

Chlorination is one of the most widely used disinfection methods in both drinking water and wastewater processing. The antimicrobial activity of chlorine is due to its oxidising capacity. There are a number of other uses for chlorine treatment in the food industry, including reduction of microbial populations on the surfaces of raw foods, such as fruit and vegetables, and sanitation of surfaces in food processing environments (Virto et al., 2005).

A reduction of 2 log₁₀ infectious units was observed after treatment of HEV-1 suspended in buffered demand-free water with 0.41 mg/L (× min) sodium hypochlorite, but up to 11.21 mg/L (× min) was needed in the presence of 1% sewage. A further 1 log₁₀ reduction was observed in the next 30 min of treatment (Girones et al., 2014). El-Senousy et al. (2014) reported the effect of chlorination on raw water and drinking water spiked with an HEV-positive sewage sample. The monitoring of residual RNA of HEV (GE) was used as a measure of chlorination treatment. In raw water, a chlorine dose of 5 mg/L for 15 min was sufficient to reduce 2 log₁₀ GE of the initial inoculum while, in drinking water, the same level of reduction of the initial inoculum was observed at the dose of 3 mg/L for 15 min, confirming that solid materials present in raw water play an important role in the protection of the virus against chlorine treatments (El-Senousy et al., 2014).

The efficacy of chlorine disinfection is influenced by several parameters, such as surface morphology, temperature and pH, and differs per type of pathogen and microbial attachment to the produce.

3.8.5.4. Other disinfectants

There are a variety of possible alternatives to NaOCl that may be effective in the reduction of viruses, such as chlorine dioxide (ClO₂), hydrogen peroxide (H₂O₂), peracetic acid (PAA), ammonia (NH₃) and ozone (O₃). The primary inactivation action of chemical disinfectants is oxidation. Several studies report on the efficacy of those commercial disinfectants against bacterial pathogens, whereas little work has been reported on the inactivation of enteric viruses, such as HEV, by such disinfectants and even less under conditions applied by the food industry. Although HEV data are lacking, results obtained with these alternative disinfection methods for other enteric viruses than HEV, including HAV and NoV, may demonstrate potential virucidal activity against HEV and thus on their applicability as control measures. These data are discussed below.

Jean et al. (2003) compared several disinfectants (10% quaternary ammonium either or not combined with 5% glutaraldehyde, 12% NaOCl, 2% iodide, 2% ClO₂) on their inactivating potential of HAV in solution and attached to common agrifood surfaces, including plastics, aluminium, stainless steel and copper. NaOCl (12%) appeared to be the most effective for HAV inactivation amongst the disinfectants tested with a maximum inactivation level of 3 log₁₀.

Bigliardi and Sane Sebastiani (2006) studied the virucidal activity of chlorine compounds using HAV and poliovirus 2, and compared these with the disinfectant efficiency of peracetic acid (PAA). HAV presented a higher resistance to HClO than poliovirus did. With ClO₂ the inactivation times of HAV were markedly shorter. PAA appeared to be less effective than chlorine.

Ozone is one of the most effective sanitisers known, which leaves no hazardous residues on food or food-contact surfaces (Khadre et al., 2001). The efficacy of ozone in inactivating NoV and feline calicivirus (FCV) in water is tested in several studies, and demonstrated inactivation by 3 and 4 log, respectively (Kim et al., 1999; Thurston-Enriquez et al., 2005; Shin and Sobsey, 2008). The results of these studies indicate that viruses can be reduced rapidly and extensively by ozone disinfection. Similar studies have to be performed to confirm the effectiveness in reducing infectious HEV as well.

The same holds true for the virucidal efficacy of liquid hydrogen peroxide and hydrogen peroxide vapour (HPV). Results on HEV inactivation are lacking, but experiments with surrogates murine norovirus (MNV) and bacteriophage φX174 demonstrated that liquid hydrogen peroxide (2.1%) was able to inactivate MNV and bacteriophage φX174 on stainless steel discs by approximately 4 log₁₀ within 10 min of exposure (Li et al., 2011a). Treatment with HPV (2.52%) resulted only in a marginal reduction (< 1 log after 5 min incubation) of these model viruses. Tuladhar et al. (2012) studied inactivation of a panel of enteric viruses, including NoV, rotavirus and adenovirus dried on to stainless steel carriers, and demonstrated that exposure to 127 ppm HPV for 1 h at room temperature resulted in complete inactivation of all viruses tested, indicating > 4 log₁₀ reduction.

Emmoth et al. (2011) studied ammonia treatment of hatchery waste and demonstrated ammonia treatment of hatchery waste to be efficient in inactivating enveloped and naked single-stranded RNA viruses. In this study feline calicivirus was used as a model virus for avian HEV, because feline calicivirus can be cultured. Although promising in their general virucidal efficacy for other enteric viruses, the effect of these alternative disinfectants on HEV inactivation needs to be studied in order to elucidate their feasibility in the control of HEV contamination and transmission. This should also take into account the effect of the quasi-envelope that may be protective for the stability of the viral particle in meat, liver or blood food products.

3.8.5.5. Hydrostatic pressure processing

Hydrostatic-pressure processing (HPP) shows great potential in the food industry for inactivating microorganisms, denaturing proteins and extending the shelf life of food products, while maintaining the quality of fresh foods, with little effect on flavour and nutritional values. In contrast to the irradiation of food, HPP is readily accepted by the consumer. HPP is industrially applied to fruit juices, jams, fruit coatings, fruit jellies, fruit desserts, avocado-based products, sliced onions, tofu and ready-to-eat vegetable dishes. Depending on the product, pressures between 400 and 600 MPa are applied, with time ranging from 3 to 30 min (for jams; Rutjes et al., 2013). Similar treatments are now commonly used for certain manufactured and ready to eat meat products (Hugas et al., 2002; Troy et al., 2016).

No information is available on reductions of HEV by HPP. However, several other enteric pathogenic viruses, such as human NoV and HAV, or surrogate viruses such as MNV have been studied for their susceptibility to HPP in several food–virus combinations. These results suggest good prospects for inactivation of enteric viruses in foods, with inactivation rates of up to $> 6 \log_{10}$ reduction for MNV in synthetic medium (Kingsley et al., 2007), $> 4 \log_{10}$ reduction in strawberry and lettuce (Lou et al., 2011), and $> 3 \log_{10}$ reduction for HAV in oysters (Calci et al., 2005). However, the efficacy of virus inactivation is not only dependent on the pressure, but also dependent on the pH and salt content of the matrix, the temperature and the strain of the virus. HAV inactivation is enhanced in acidic matrices, whereas MNV-1 and hAdV2 were shown to be more sensitive to HPP at neutral pH than at acidic pH (Lou et al., 2011; Kovac et al., 2012). Temperature also has a great impact on the efficacy of HPP. Chen et al. (2005) reported that temperatures above and below 20°C significantly increased HPP inactivation of FCV, and Lou et al. (2011) reported that MNV-1 was more effectively inactivated at 4°C than at 20°C. HPP studies performed with human pathogens and surrogates in food have been reviewed by Rutjes et al. (2013).

In conclusion, HPP appears to be effective in reducing enteric viruses in food. However, considering the unpredictable effects of pH, temperature and matrix on virus reduction, it is difficult to extrapolate results from surrogate viruses to HEV.

3.8.5.6. Irradiation

Irradiation in the food industry has been established as a safe and effective method for food processing and preservation. Besides being used for reduction of pathogenic microorganisms, it is also applied for the prevention of food spoilage and sprouting, and delaying of fruit ripening (Farkas, 1998). Irradiation may cause a change of texture or colour in fruit and vegetable tissues, this being one of the main limiting factors in its use on fresh produce (Han et al., 2004).

Three different types of ionising radiation are currently available and used for the irradiation of food: gamma rays (γ -rays), X-rays and electrons (e-beams).

Two of the types of ionising radiation techniques are produced by converting other energy sources, such as electric current without the involvement of radioactive substances. e-Beams consist of a stream of high-energy electrons accelerated by specific equipment that converts electricity and can be switched on and off depending on the need. X-rays are a further evolution of e-beams (EFSA BIOHAZ Panel, 2011b).

In the EU, 12 MS have, within their territory, a total of 23 irradiation facilities approved for irradiation of food. Approvals are granted by the competent authorities in MS, in accordance with the procedure established by EU Directive 1999/2/EC.⁹ Foodstuffs and doses (temporarily) admitted at MS level have been published by the Commission (EU Directive 1999/3/EC and OJ C 283/02, 24.11.2009, p. 5). The foodstuffs that are included in this list and may play a role in transmission of HEV to

⁹ Directive 1999/2/EC of the European Parliament and of the Council of 22 February 1999 on the approximation of the laws of the Member States concerning foods and food ingredients treated with ionising radiation. OJ L 66, 13.3.1999, p. 16–23.

humans are limited, and include (dried) vegetables and pulses, soft fruit, chicken and poultry, meat and shellfish. Pig meat and other pig products are not listed in this EU regulation.

Irradiation is an effective method for food decontamination (Beuchat, 1998). It has been shown that vegetative organisms can be easily destroyed by radiation. However, additional data are needed on the effect of ionising radiation on virus reduction in food (Tewari, 2003). Viruses are, in general, more resistant to radiation treatment as compared with bacteria (Shea, 2000; De Roda Husman et al., 2004; Fino and Kniel, 2008). The mode of action for virus inactivation is mainly based on the reaction of hydroxyl radicals with nucleic acid strands. The virus coat may also play a role (De Roda Husman et al., 2004), as well as direct cleavage of DNA or RNA (Feng et al., 2011). Damage can also occur indirectly because of the interaction of radiation with matrix molecules adjacent to genetic material, which may cause similar damage to those radicals resulting from direct radiation (FAO/IAEA/WHO, 1999; Dickson, 2001; Ahn and Lee, 2006).

Little information is available in the scientific literature on the effects and efficacy of virus inactivation by e-beam and X-ray radiation, and limited research has been done using γ -radiation. No data are available on inactivation of HEV by any of the above-mentioned irradiation types. Several studies on the inactivation of NoV and surrogate viruses such as feline and canine calicivirus using γ -radiation in water have been described. However, no direct conclusions from these results can be drawn for the inactivation of these viruses in food, because γ -radiation is less effective in the presence of scavengers present in foods, which react with hydroxyl radicals (e.g. proteins, cellulose and polysaccharides). Sullivan et al. (1973), for instance, studied the resistance of the enteric Coxsackievirus A9 to γ -irradiation in water and ground beef, and reported that the values necessary to achieve 1 \log_{10} reduction varied between 1.4 kGy and 7.6 kGy, respectively. Feng et al. (2011) demonstrated that γ -radiation at the FDA-approved dose of 4.0 kGy to control food-borne pathogens in fresh iceberg lettuce and spinach does not effectively inactivate MNV-1 in fresh produce. For MNV-1, a 1.7–2.4 \log_{10} virus reduction was achieved in spinach, lettuce and strawberries at a dose of 5.6 kGy. Bidawid et al. (2000) studied the effect of γ -radiation on HAV present on lettuce and strawberries. Doses of 2.72 and 2.97 kGy were necessary to reduce HAV populations by 1 \log_{10} on lettuce and strawberries, respectively. Besides types of pathogen and food commodity, temperature has a great impact on microorganism inactivation efficacy because of the higher activity of produced radicals (Krämer, 2002). Furthermore, pH and temperature of water content determine the radiolytic products formed during irradiation and thus its effectiveness (Diehl, 1995). Irradiation can, however, cause a change of texture or colour in fruit and vegetable tissues, this being one of the main limiting factors in its use on fresh produce (Han et al., 2004).

Within the EU, irradiation is allowed only in a limited number of foodstuffs that are of relevance for transmission of HEV, which do not include pig meat or pig products. However, for fresh produce, such as leafy green vegetables and soft fruit, as well as for shellfish, irradiation may reduce HEV levels to some extent, assuming that HEV inactivation is similar to the analysed enteric viruses and their surrogates. Although no data are available on the inactivation of HEV by irradiation, it is to be expected that the effect of food irradiation on the reduction of food-borne transmission of HEV is limited taking into account that irradiation of pig meat and other pig products is not allowed within the EU. A drawback of food irradiation technology is lack of consumer acceptance, because of consumers' fear of induced radioactivity and the consequent unwholesomeness of irradiated foods (Resurreccion et al., 1995).

3.8.5.7. Development of innovative meat treatments

Curing has been the dominant method of meat preservation for thousands of years, although modern developments like refrigeration, synthetic preservatives and innovative treatments (i.e. quick-dry-slice process) are now beginning to complement and supplant it, reducing the time of food processing. Some popular processes for curing of pork products included addition of salts and nitrates and long drying periods producing denaturalisation of proteins and a final product where viruses could not survive. Over recent years a progressive reduction in the time of curation in meat products has occurred, and quick systems have been implemented that could adversely influence the level of survival of viruses at the point of consumption (Edwards, 2000).

The safety of each reformulated food should be evaluated on a case-by-case basis considering microbial pathogens and specifically HEV in the animal reservoirs, and following the HACCP-based approach (Stringer and Pin, 2005; Taormina and Taormina, 2010).

3.8.5.8. Data gaps for the control of HEV in food treatment processes

The review of the literature highlighted numerous data gaps on HEV persistence in food, which must be filled to implement its control. The lack of an efficient cell culture system has hampered collection of data on the virus, its occurrence, persistence in food and, most importantly, its resistance to control measures.

At present, there is information on food products in which (infectious) HEV is regularly detected, but there is also a lot of uncertainty on the products that may be contaminated by blood products such as meat glue. In addition, the prevalence of HEV in foods of non-animal origin, such as fruit and vegetables and shellfish, has not been investigated in detail. Moreover, the concentration of HEV in/on the contaminated products is not easy to establish, partly due to the large variety of methods that are currently applied to detect viruses in food products. Several molecular detection methods have been described, and most of those methods have shown that they are able to detect HEV in one or more foodstuffs. However, results are dependent on sampling, sampling size, extraction method, inhibition, etc., which all impact the accuracy of quantification and thus comparability of the results. Without an efficient cell culture system, information on the infectivity of the detected virus is missing as well as the possibility to study the efficacy of treatment processes.

Without accurate quantitative data on the concentrations of infectious HEV in food, a reliable assessment of required reduction levels by treatment processes is difficult. Furthermore, without an efficient cell culture system, information on the efficiency of existing measures to control virus contamination during production and processing will be difficult to obtain for HEV. Mapping the foods of animal- and non-animal origin that may be contaminated by HEV may help to prioritise the treatment processes that need to be studied for their inactivating capacities.

No data are available on the resistance of HEV under food-processing technologies such as curing, drying and smoking, which are processes involved in production of some food of animal origin consumed raw and so place consumers at a higher risk.

Without an efficient cell culture system for the detection of HEV, the identification of a reliable surrogate virus would assist in estimating the efficiency of HEV inactivation by different treatment processes. Several surrogate viruses, such as family members of the *Caliciviridae* (feline or canine calicivirus, MNV), *Picornaviridae* (HAV, polio- or other enteroviruses), adenoviruses and bacteriophages, such as MS2 have been proposed.

Although there will be similarities between the surrogates and HEV, there are also limitations of using surrogates. For example, FCV is a respiratory pathogen and not very stable at low pH; MNV, used as model for human NoV, has a genome organisation similar to HEV but a probable different tropism in the host. HAV, like HEV, does not have an envelope, but acquires one when it grows in the hepatocytes during replication in the liver (Feng et al., 2013). Of the surrogate viruses, HAV has shown the greatest resistance to heat, desiccation, extreme pH and ionising radiation, so it may be considered a conservative surrogate for HEV when studying certain treatment processes (FAO/WHO, 2008). Thus, although, preferably, experiments should be performed using HEV, in the absence of a sensitive culture system for HEV, the second best option would be to use surrogates. More information on the suitability of a surrogate for HEV is required to be able to translate inactivation of these surrogates to inactivation of HEV.

Concluding remarks

- The lack of an efficient cell culture system and the disadvantages of the use of animals have made it difficult to achieve a clear result on the effect of heat treatment on HEV survival. More studies are needed to evaluate the residual infectivity and the inactivation kinetics of HEV after thermal treatments (heating and long thermal storage).
- Different combinations of temperature and time are effective in the inactivation of HEV-3, dependent on the used matrix: More than 3.5 log infectivity reduction of cell-culture-adapted HEV was achieved by heating at 80°C for 1 min or at 70°C for 2 min. Boiling or stir-frying of HEV positive liver samples (internal temperature 71°C) for 5 min showed no residual infectivity in pigs, whereas heating at 71°C for 20 min was necessary in pâté-like preparations.
- Only a few studies on HEV in bivalve molluscs are available, yet persistence of HEV cannot be ruled out in shellfish consumed raw or lightly cooked.
- Limited information is available on the effect of biocidal treatments, disinfection and HPP applied in the food industry on the infectivity of HEV.

- Some types of radiation (γ -rays) effectively reduce viral load within food, but this has not yet been investigated for HEV. Its use is restricted by European legislation and by a lack of consumer acceptance.
- HEV is sensitive to current water disinfection treatments using chlorination and UV irradiation, similar to other viruses. These measures can be also used to minimise cross-contamination through treatment of food contact surfaces, decontamination of water for irrigation or shellfish mussel depuration.

3.8.6. Reduction of HEV in the environment by applied treatment processes

Pig manure and wastewater from humans are the main sources of HEV in the environment. Due to more restricted legislation for the application of manure and manure products, manure treatment processes are increasingly applied. Treatment processes are mainly required for the reduction of phosphates and/or processing into new products, but they also impact on the concentration of infectious pathogens. Commonly applied manure treatments are reverse osmosis, composting, fermentation and pasteurisation. The efficiency of HEV reduction by reverse osmosis has been studied in six manure treatment plants in the Netherlands (Hoeksma et al., 2015). Comparing HEV RNA concentrations in the different fractions, the liquid and solid manure fractions and concentrate fractions contained about 4 log₁₀ HEV RNA particles per gram. In the permeate, the liquid fraction that is discharged into surface waters, a 4 log₁₀ reduction of HEV RNA particles was observed; in four of the six permeate fractions that were analysed, no HEV RNA was detected. Somatic coliphages were not detected in these permeate fractions, indicating that > 5 log reduction was achieved. Treatment of pig manure by mesophilic fermentation reduced somatic coliphages by 1.4 log. After subsequent pasteurisation (1 h at 70°C), no somatic coliphages were detected, thus > 5 log reduction was observed by the combination of fermentation and pasteurisation (Hoeksma et al., 2015).

In Spain, the presence of HEV in five pig manure composting plants was evaluated. A total of 594 samples were taken in 54 sampling sessions from the different stages of composting treatment in pig manure composting plants located in Spain as follows: slurry reception ponds, anaerobic ponds, aerobic ponds, fermentation zone and composting final products. HEV was detected by RT-nPCR in four out of five plants studied, mainly in the first stages of the process. HEV was not detected in any final product (compost) samples, destined to be commercialised as a soil fertiliser, suggesting that composting is a suitable method to eliminate HEV, probably because of the generation of heat (Barnaud et al., 2012) and microbial competition (Garcia et al., 2014).

In a French study, including three herds with different effluent treatment, 67% of the untreated pig slurry samples were positive for HEV. After treatment, 27% of pig slurry was still positive for HEV. Among those, 30% of treated pig slurry was positive for HEV after composting, 50% after dehydration, and only 5.6% of the pig slurry treated by anaerobic digestion was positive for HEV (Loisy-Hamon and Leturnier, 2015).

In raw surface water samples inoculated with HEV viruses, 5 mg/L chlorine for 15 min was sufficient to reduce HEV quantified by qRT-PCR by 1 log₁₀, and for samples inoculated after autoclaving the reduction was 2 log₁₀, while in drinking water samples 3 mg/L for 15 min was sufficient to achieve a 4 log₁₀ reduction and 4 mg/L produced a 6 log₁₀ reduction of HEV (El-Senousy et al., 2014).

Riverbank filtration methodologies can substantially reduce the release of infectious viruses into rivers (Sprenger et al., 2014). Low rainfall during summer can lead to higher concentrations of human viruses, and river and seawater present the highest viral concentrations during warmer months. Alternatively, higher temperatures will cause an increased inactivation of the virus (Schijven and de Roda Husman, 2005). In a global context, wastewater management will be key to preventing environmental dispersion of human and animal faecal pathogens in future climate change scenarios (Rusinol et al., 2015), and efficient wastewater treatments such as submerged membrane bioreactor technology may offer improved removal of viral contaminants (Hmaied et al., 2015). UV or flocculation–chlorination techniques can also be used to help safeguard drinking water (Girones et al., 2014; Guerrero-Latorre et al., 2016).

Concluding remarks

- In relation to environmental sources of HEV, various manure composting and waste treatment regimens have been shown to have the potential to reduce environmental contamination by HEV, but wider studies analysing loss of HEV infectivity by treatment processes are required before firm general recommendations can be made.

3.9. Microbiological control measures and protection at the consumer level

As defined by the European Commission, microbiological criteria give guidance on the acceptability of foodstuffs and their manufacturing processes. Preventative actions, such as the application of GHP and GMP, and the Hazard Analysis Critical Control Point (HACCP) principles contribute to achieving food safety. Microbiological testing alone cannot guarantee the safety of a foodstuff tested, but these criteria provide objectives and reference points to assist food businesses and competent authorities in their activities to manage and monitor the safety of foodstuffs, respectively.¹⁰

Commission Regulation (EC) no 2073/2005 on microbiological criteria for foods, applicable from 2006, lays down food safety criteria for relevant food-borne bacteria, their toxins and metabolites, such as *Salmonella*, *Listeria monocytogenes*, *Cronobacter* (before *Enterobacter*) *sakazakii*, staphylococcal enterotoxins and histamine in specific foods; however, there are no safety criteria considering food-borne zoonotic viruses such as HEV. These criteria would define the acceptability of a product or a batch of food applicable to products placed on the market. Until now, no specific European Commission legislation including microbiological criteria exists for viruses in fresh produce or meat, and no specific legislation for HEV is currently in place.

Regulations related to hygienic measures for foods of animal origin and control of products of animal origin for consumption are laid down in EU legislation 853/2004 and 854/2004, and ante-mortem and post-mortem inspections are not efficient tests for detection of HEV that may be present in liver or meat at the time of slaughter. The presence or absence of bacterial pathogens is not an indication of the presence of HEV in meat products. It is important also to note that HEV will be very stable at the low temperatures used for preservation of meat products.

Methods exist for detecting viruses in fresh produce, shellfish and meat, and in environmental sources of HEV. New information is currently being produced on wastewater and manure treatments for reducing pathogens, and on the transmission of HEV in animal reservoirs. The development of validated quantitative and qualitative detection methods, including infectivity assays, and consensus molecular typing protocols is required for the development of quantitative microbial risk assessments and efficient control measures.

Hepatitis E may now be considered an anthrozoosis as well as an anthroponosis. Pigs, wild boars and deer have been identified as reservoirs, and their flesh and entrails (consumed as meat and offal) as vehicles of HEV transmission. Dietary, gastronomic and culinary preferences influence how extensively HEV conveyed by these vehicles can be inactivated before their ingestion by the host. Another route of infection is via HEV that is faecally shed by humans and by live animals into the environment (Teo, 2010), and may thereby contaminate vegetables, fruit or shellfish.

There are several studies that identify consumption habits, consumption of raw or undercooked pork or wild boar products (e.g. sausages, salami), as risk factors for HEV infection in developed countries, particularly in Europe (Tarantino et al., 2016). A study by Mansuy et al. (2016) showed that seroprevalence varies even within a single country, and that HEV is highly endemic in some areas of southern and north-eastern France, with dietary habits being a major factor in the transmission of HEV (Figatelli in the south, Pate à quenelle in the north-east). However, consumption of bottled water had a protective effect, suggesting that contaminated water could also be involved in the epidemiology of HEV infections in France. Seroprevalence data indicated considerably lower numbers of cases of HE in northern and southern European countries (Adlhoch et al., 2016) and this will be mainly related to consumption/preparation habits. In order to develop risk assessment studies, individual tastes, cooking habits, gender, socioeconomic background and other factors are very likely to influence meat consumption and hence the risk of contracting HEV infection and of developing acute disease (EFSA BIOHAZ Panel, 2011a). A recent study (Muller et al., 2017) for the quantification of the risk of hepatitis E for Swiss consumers by specified pork products, making assumptions for the hazard characterisation due to the lack of a dose-response relationship for oral exposure to HEV and considering only products containing pork liver, showed a significant risk level, the most likely annual number of food-borne hepatitis E cases in Switzerland was estimated to be 1,481 (95% CI 552; 4,488) if all products containing pork liver were considered; if only high-risk products, such as plain pork liver and liver sausages (e.g. Saucisse au Foie), were considered, the annual number of cases was estimated to be 176 (95% CI 64;498).

¹⁰ http://ec.europa.eu/food/safety/biosafety/food_hygiene/microbiological_criteria_en

As it is also stated in the report of the EFSA Panel on Biological Hazards (BIOHAZ), Scientific opinion on an update on the present knowledge on the occurrence and control of food-borne viruses (2011), presently the only efficient control option for HEV infection from consumption of meat, liver and products derived from animal reservoirs is sufficient heat treatment. General guidance for heat treatment of risk products at the production and consumption levels could be developed, although the precise time/temperature conditions for inactivation of HEV in meat products are not well known and more information is needed before specific requirements or recommendations for the diverse food products are put in place. Improved kitchen and butchery hygiene may help prevent transfer of HEV from raw meat to products thereafter eaten raw.

A useful initiative for prevention of HEV infections is the implementation of education campaigns, especially for the meat industry and butcheries and for consumers within risk groups. Thorough cooking of meat, liver and meat products derived from wild boars and pigs could be recommended for high-risk consumer groups. More specific recommendations of protective measures to minimise the risk of HEV infection from food of animal origin are: (i) wash hands after handling food products that may have a higher risk of having HEV virus (uncooked liver and liver products and meat); (ii) wash utensils with hot soapy water after preparing a food item with raw meat or liver; (iii) if possible, use one cutting board for fresh produce and a separate one for raw meat or liver; (iv) as it is generally recommended never place cooked food on a plate that previously held raw meat; (v) very important: cook pork, wild boar and deer meat and products thereof to a safe minimum internal temperature of 71°C for 20 min.

Concluding remarks

- There are several studies that identify consumption habits, consumption of raw or undercooked pork or wild boar products (e.g. sausages, salami), as risk factors for HEV infection in Europe.
- The development of validated quantitative and qualitative detection methods, including infectivity assays, and consensus molecular typing protocols is required for the development of quantitative microbial risk assessments and efficient control measures.
- In order to minimise the risk of an HEV infection, consumers should thoroughly cook especially pork and wild boar meat products. This recommendation applies in particular to especially vulnerable groups (e.g. persons with a weakened immune system, pre-existing liver injury).

4. Conclusions

4.1. ToR 1: to critically review current methods for the detection, identification, characterisation and tracing of HEV. In the case of methods for detection, special emphasis should be made to assess their application to samples from food-producing animals and foods

- A diversity of methods for HEV extraction and RNA purification from animals and a range of food and water samples are available; however, standardisation, systematic method comparison and interlaboratory validation has not been performed for most virus extraction methods. Meat and meat products are considered to be high priority for method development.
- Quantification protocols for HEV, based on various (real-time) RT-PCR-based and LAMP-based techniques for the detection of the HEV genome, have been developed, although no standardised assays for use with animal, food or water samples are available so far.
- Several methods for typing and subtyping of HEV strains and for source attribution and tracing are available. However, the methods are not yet harmonised or standardised, leading to incongruences in subtyping of strains. The recent definition of HEV subtype reference strains, a distinct set of whole genome reference sequences for HEV-1 to HEV-7 subtypes and the current development of the web-based typing tool 'HEVnet' represent important steps towards harmonisation. Thresholds for definition of types, subtypes and 'identical' strains based on sequence comparisons remain to be agreed.
- Several tests available for detection of IgM or IgG in human and animal sera have been established. However, as test characteristics vary between the different serological assays it is important to consider these when the results are interpreted.
- ELISA/EIA as a direct test for HEV antigen detection in serum or faeces correlates well with quantitative detection of HEV RNA in matrices tested, although it shows lower sensitivity.

- Numerous descriptions of HEV isolation in cell culture exist, although isolation from low-HEV-concentration samples is often unsuccessful and only a few studies have tested the assays with regard to reproducibility and sensitivity. Reports on HEV isolation from food samples in cell culture are rare, and no standardised or validated method for preparation of food samples before inoculation into cell cultures is available.
- Pig or monkey inoculation models can be used for HEV infectivity assessment, including the analysis of food samples. However, the application of animal inoculation models is restricted by the unknown applicable range of virus concentrations, the limited potential for sample replication, ethical considerations, and the laborious, time-consuming and expensive nature of large animal experiments.
- Alternative methods for indirect estimation of HEV infectivity are so far restricted to a capsid integrity assay. The assay has not been validated by comparison with direct infectivity assays such as cell culture.

4.2. ToR 2: to review the scientific literature on HEV reservoirs (human, animal and environmental) focusing on genotypes of public health significance in order to determine the importance of food-borne pathways and identify potential control options

- The species *Orthohepevirus A* includes two genotypes of HEV originating from humans only (HEV-1 and HEV-2) and two genotypes reported from both humans and different animal species (HEV-3 and HEV-4). The latter are associated with food-borne infections linked to pigs, wild boar and deer meat. Other recently described closely related strains with more limited public health relevance have been found in a range of animals including wild boars (HEV-5 and HEV-6), rabbits (HEV-3ra) and camels (HEV-7 and HEV-8).
- In EU/EEA, genotype 3 viruses with the most common subtypes being HEV-3c and HEV-3e, f, g, and very few cases of HEV-4, have been described in humans. Molecular evidence of the source of transmission and relationship of viruses is provided by studies that compared virus sequences and subtypes derived from human cases with viruses from pigs, wild boar and deer or consumed products thereof.
- Food-borne transmission appears to be the major pathway for human HEV infections in Europe. Raw or undercooked pork meat or pork liver sausages are the most frequently reported food products associated with sporadic cases or outbreaks of HEV. Domestic pigs are the main animal reservoirs of HEV in the EU. Wild boars are also an important reservoir, but their population is lower compared to pigs and wild boar meat is less commonly consumed. Contact with infected animal reservoirs is also a risk for HEV infection. Personnel with occupational exposure to pigs or wild boar are more frequently seropositive than the general population.
- HEV-infected pigs have been identified at the farm and abattoir level in all countries where investigations have been carried out. Prevalence of HEV varies greatly between farms, production systems and countries, with small non-industrialised production being at higher risk in some studies.
- A proportion of pigs, likely to be less than 10%, remain viraemic at slaughter, which is a probable cause of prime meat cuts containing HEV.
- Studies in some countries suggest that HEV-free pig herds may exist, but this should be confirmed by more intensive testing.
- Cumulative genetic and epidemiological evidence from several countries where wild boars are prevalent suggests that HEV infection is common and poses a zoonotic hazard for hunters and consumers of wild boar meat.
- Deer meat may present a zoonotic risk; however, the HEV prevalence in deer is comparatively low. There appears to be a statistical association between the prevalence of HEV in wild boar and deer populations in some regions.
- A few host-associated HEV strains of rabbits (HEV-3ra) appear to have close sequence homology with some strains found in people, but the relative importance of natural transfer of HEV from rabbits to people needs to be determined. Antibodies to HEV and RNA have been found in farmed, wild and laboratory rabbits in several countries, but rabbits appear to be resistant to infection with HEV-3 strains detected in humans and pigs.

- There is no evidence of HEV infection in goats, sheep and cattle in Europe. However, specific antibodies to HEV have been found in other countries, especially in areas where there is heavy environmental exposure to HEV. More data are required to clarify the reservoir status of these animal species for zoonotic strains of HEV.
- HEV-7 has been detected once in an immune-compromised patient exposed to camels. The zoonotic potential of HEV-7, and a proposed HEV-8, and the risk of transmission from camel to humans remain to be elucidated.
- Various animals, such as moose, rats, ferrets, bats and several species of birds, have been reported to carry host-specific variants of HEV but there is currently no evidence for zoonotic transmission of HEV from these.
- Contamination of the environment with HEV from human and animal faecal waste may lead to contamination of drinking water, fruit and vegetables, and bivalve molluscs. HEV-3 infectivity was detected up to 28 days at room temperature in separated cell culture media suggesting that the virus could persist for several weeks in the environment. However, there are significant knowledge gaps on the survival of HEV and the effect of decontamination procedures used in primary production and food chain processes. The lack of a reliable infectivity assay or surrogate model has hindered such studies.

4.3. ToR 3: to examine currently available information on the geographical distribution of HEV and epidemiology, occurrence and persistence in foods and consumer habits contributing to infection

- Hepatitis E is an important infection in humans in EU/EEA countries causing more than 21,000 mostly locally acquired reported clinical cases with 28 fatalities over the last 10 years with an increasing trend. However, as infection in humans is not notifiable in all MS, and surveillance differs between countries, the number of reported cases is not comparable and the true number of cases would probably be higher.
- Outbreaks and sporadic cases have been identified in immune-competent persons as well as in recognised risk groups such those with pre-existing liver damage, immunosuppressive illness or receiving immunosuppressive treatments.
- Immunosuppressed patients are at risk of developing chronic HEV infections with severe disease progression and fatal outcomes. A few human-to-human transmission events have been blood- and transplant-related.
- Occupationally exposed humans having contact with the animal reservoirs show higher seroprevalence than the general population, providing evidence for zoonotic transmission from animals to humans; vegetarians show lower seroprevalence. Outbreak investigations and molecular studies have identified the same virus strains in reservoir animals, food and infected humans.
- Pork meat is the major type of meat produced in the EU-28. The wild boar population is growing however, compared to pork meat, the amount consumed is much smaller.
- HEV infection in pigs is largely subclinical, but minor hepatitis has been reported after experimental infection. The duration of viraemia and faecal shedding of the virus is variable according to the management system, the age at first exposure and opportunities for recycling of infection.
- Transmission of HEV from infected sows to piglets and trade in carrier pigs has been responsible for wide dissemination of infection. Specific HEV strains can persist for long periods on pig farms, but a turnover of strains can also occur.
- Infected animals are carriers of the virus that can be shed at a high level in faeces and bile, and can be found at a lower level in meat. This could represent a possible vehicle for food cross-contamination during slaughter, evisceration and food processing or handling.
- HEV has been reported in food of animal origin both at the slaughterhouse and point of sale. Products containing raw liver have been frequently found to be HEV RNA-positive.
- HEV infection is mainly linked to consumption of raw or under-cooked virus-contaminated liver and processed meat products.
- Blood from viraemic animals could be a potential source of HEV infection if used in food products (e.g. blood sausages) that are insufficiently cooked.

- Other blood-derived products such as fibrinogen are increasingly used as ingredients in meat and also in non-meat processed foods and dietary supplements and may constitute a risk only if not properly heat-treated.
- The role of the environment (e.g. organic fertilisers or irrigation water) as source for HEV contamination of food of non-animal origin remains to be evaluated.
- A small number of studies have reported a low prevalence of HEV in shellfish, soft fruit and vegetables.
- There are several studies that identify regional consumption habits such as consumption of raw or undercooked pork or wild boar products (e.g. sausages, salami), as risk factors for HEV infection in Europe. There are indications that the human population of Central European countries have a higher seroprevalence than, for example, Nordic populations.

4.4. ToR 4: to investigate possible control measures along the food chain and evaluate decontamination treatments

- Present control measures in the pork production chain are based on prevention of certain meat-borne parasitic or bacterial zoonoses and are not sufficient to prevent presence of HEV in pork.
- Regulations related to hygienic measures for foods of animal origin and control of products of animal origin for consumption are laid down in EU legislations 853/2004 and 854/2004. Ante-mortem and post-mortem inspections are not able to detect HEV, which may be present in liver or meat at the time of slaughter.
- Some management-based methods such as batching of farrowing and weaned pigs to influence the time of first exposure to HEV and farm decontamination measures for potential reduction of HEV risk in pig herds have been described, but need to be confirmed in well-designed intervention studies.
- A recombinant subunit vaccine, based on a HEV-1 peptide strain, was registered for humans in China in 2011, yet has not been licensed or approved in other countries. Vaccination of pigs is a potential control option, but the effect of vaccination of pigs as a possible method to prevent human infection requires further investigation. No vaccine for animals is commercially available currently.
- Control options mentioned in EFSA Scientific Opinions for NoV and HAV could also be beneficial for HEV.
- Viral HE particles can exist as non-enveloped or 'quasi-enveloped' virions, and the envelope may also influence the stability of the viral particle in meat, liver or blood food products.
- The lack of an efficient cell culture system and the disadvantages of the use of animal infectivity models have made it difficult to achieve meaningful data on the effect of heat treatment on HEV survival. More studies are needed to evaluate the residual infectivity and the inactivation kinetics of HEV after thermal treatments and storage.
- Different combinations of temperature and time are effective in the inactivation of HEV-3, dependent on the used matrix: More than 3.5 log infectivity reduction of cell-culture-adapted HEV was achieved by heating at 80°C for 1 min or at 70°C for 2 min. Boiling or stir-frying of HEV-positive liver samples (internal temperature 71°C) for 5 min showed no residual infectivity in pigs, whereas heating at 71°C for 20 min was necessary in pâté-like preparations.
- Only a few studies on HEV in bivalve molluscs are available, yet persistence of HEV cannot be ruled out in shellfish consumed raw or lightly cooked.
- Limited information is available on the effect of biocidal treatments and disinfection, or hydrostatic pressure processing applied in the food industry on the infectivity of HEV.
- γ -Ray irradiation reduces viral load within food, but this has not yet been investigated for HEV. Its use is restricted by European legislation and by a lack of consumer acceptance.
- HEV is sensitive to current water disinfection treatments using chlorination and UV irradiation, similar to other viruses. These measures can be also used to minimise cross-contamination through treatment of food contact surfaces, decontamination of water for irrigation or shellfish mussel depuration.
- Various manure composting and waste treatment regimens have been shown to have the potential to reduce environmental contamination by HEV, but wider studies analysing reduction of HEV infectivity by treatment processes are required before general recommendations can be made.

5. Recommendations

- The validation and standardisation of methods for detection and quantification of HEV from meat and meat products should be a high priority. Also, detection methods for other food matrices (e.g. shellfish, fruit and vegetables, food contact surfaces) and bottled water as described in ISO15216 should be validated in order to demonstrate their suitability for the detection of HEV.
- The development of efficient cell culture methods for HEV should be encouraged, including their optimisation with regard to reproducibility and sensitivity.
- There is a need for harmonisation of typing, subtyping and strain comparison methodologies. Thresholds for definition of types, subtypes and 'identical' strains based on sequence comparisons should be defined.
- The databases used in web-based typing platforms should be encouraged to include strains from animals, food and the environment in order to support epidemiological investigations.
- Studies are needed to quantitatively estimate the level of contamination in foods of animal origin, including foods other than those containing pig liver that have rarely been investigated, and to determine the correlation of HEV RNA detection with infectivity of the virus.
- Evaluation of the potential impact of vaccination of pig herds against HEV and other options such as HEV-free pig herds, and the impact of management methods should be carried out.
- Data on survival of HEV in meat products, bivalve molluscs, fruit and vegetables and their production and processing environment are needed. The risk of transmission of HEV from contaminated water to food should be determined.
- Despite considerable research in recent years, the level of awareness of HEV risk associated with pig meat products and other reservoirs and sources is low, so dissemination of information and advice to consumers and those working with potential sources of infection should be optimised. In particular, provision of information on the risk of consumption of raw or undercooked pig, wild boar and deer products to vulnerable groups (e.g. persons with a weakened immune system or pre-existing liver damage) may help prevent the most serious HEV infections.
- Considering the high concentrations of HEV detected in pork liver, those who produce and market foodstuffs with pork liver should take preventive measures to minimise risk of HEV transmission to consumers.
- In order to minimise the risk of an HEV infection, consumers should thoroughly cook meat and offal, especially pork, wild boar and deer meat products.

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Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
CD4	cluster of differentiation 4
CI	confidence interval
ECDC	European Centre for Disease Prevention and Control

EEA	European Economic Area
EIA	enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FCV	feline calicivirus
FDA	Food and Drug Administration
FFU	focus forming unit
GAP	Good Agricultural Practices
GE	genome equivalents
GHP	Good Hygiene Practices
GMP	Good Manufacturing Practices
HACCP	Hazard Analysis Critical Control Point
HAV	hepatitis A virus
HE	hepatitis E
HEV	hepatitis E virus
HPP	hydrostatic pressure processing
HPV	hydrogen peroxide vapour
Ig	immunoglobulin
ISO	International Organisation for Standardisation
IU	international unit
LAMP	loop-mediated isothermal amplification
MNV	murine norovirus
MS	Member State
NAT	nucleic acid amplification technique
NGS	next-generation sequencing
NoV	noroviruses
nPCR	nested polymerase chain reaction
OR	odds ratio
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PRRSV	Porcine reproductive and respiratory syndrome virus
RGE	RNA-protected genome equivalent
RNA	ribonucleic acid
RT-LAMP	reverse transcription loop-mediated isothermal amplification
RT-PCR	reverse transcription polymerase chain reaction
RT-qPCR	reverse transcription real-time polymerase chain reaction
SDPP	spray-dried porcine plasma
SPF	specific-pathogen-free
SMRT	single-molecule real-time technology
TCID ₅₀	50% Tissue culture Infective Dose
TFL	time to first log reduction
TVC	total viable count
UV	ultraviolet
WB	western blot
WG	Working Group