Stability and inactivation of hepatitis E virus at different physico-chemical treatments including pH, salt, high pressure and drying

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Declaration of Independence

Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me.

Alexander Hans Wolff Berlin, September 2, 2022

Table of contents

1 Summary

1.1 Summary in English

The hepatitis E virus (HEV) can cause acute or chronic liver inflammation in humans. In the last two decades, the numbers of notified hepatitis E cases have increased sharply, also in Germany. Here, the HEV genotype 3, which is also widespread in domestic pigs and wild boars, plays the most important role. For this genotype, the consumption of contaminated meat products from infected animals is assumed to be the main route of transmission. However, other transmission routes, such as via animal contact, contaminated surfaces or blood products, can also play a role. To assess the risk potential of specific foods and transmission routes, knowledge on the HEV stability and inactivation is an important prerequisite; however, this knowledge was largely missing at the beginning of the studies presented here.

Therefore, the aim of the work was to determine basic data on the stability of HEV at different physico-chemical conditions such as pH, salt concentration, drying as well as a treatment with high pressure. Priority was given to investigate conditions, that are used in food production or that can be expected after contamination of surfaces, in order to be able to draw practical conclusions.

For the investigations, HEV preparations in buffer solutions were subjected to the appropriate treatments and the residual infectivity was subsequently determined by cell culture cultivation and immunofluorescence analysis of viral proteins. After optimization of the method, virus amounts of approximately 10^4 infectious units per ml could be used, and their complete inactivation was considered as sufficient based on similar requirements for disinfectant testing.

In the first study, the stability of HEV against different pH values was investigated. Here, it was determined that efficient inactivation after 3 hours of incubation at room temperature (RT) occurred only at pH 1 and pH 10, whereas the virus was stable against pH 2 - 9. Further experiments were performed with the addition of D/L-lactic acid in the range between pH 4.5 and 6.5, in order to simulate conditions during food production and preservation. Here, only a slight reduction in HEV infectivity was found after 7 days at RT, whereas no significant HEV infectivity reduction was

detected at 4 °C under the same conditions compared to the respective control at pH 7.7.

In the second study, the salt stability of HEV was investigated. At sodium chloride concentrations of up to 20%, no reduction in HEV infectivity was detected after 24 hours at RT compared to the control. Salt conditions, as used in meat preservation, also elicited only minor reductions in HEV infectivity after 6 days at RT or after 8 weeks at 16 °C compared to the respective control samples without salt addition.

In the third study, the high pressure stability of HEV was investigated. Here, it was shown that HEV was almost completely inactivated after 2 minutes at 600 MPa, which represents the highest pressure commonly used for food preservation. Lower pressures resulted in smaller decreases in infectivity. Longer pressure holding times and lower temperatures resulted in stronger decreases in HEV infectivity.

In the fourth study, the drying stability of HEV on different surfaces was investigated. Here, the infectivity of HEV decreased only slightly due to the drying process. During subsequent storage at RT and 26% relative humidity, complete HEV inactivation was achieved only after 8 weeks. At 3 °C and 98% relative humidity, very high HEV concentrations were still found on plastics after 8 weeks, followed by ceramics and steel, whereas the virus was completely inactivated on wood at this time point.

It can be concluded from the results that HEV is very stable against physico-chemical treatments. Under conditions such as those prevailing in raw sausage production, HEV cannot be inactivated efficiently. Therefore, raw sausage products must be considered as potential source of infection. High pressure treatment of risk foods could be an effective measure to increase food safety. Since HEV is exceptionally stable after drying on surfaces, strict hygiene measures during food preparation and in the environment of patients are prerequisites for the interruption of infection chains. In summary, the obtained stability data provide basic insights into the properties of the HEV particle, which can be relevant for applied aspects in food production, but also for other areas such as medical products or hospitals.

1.2 Zusammenfassung auf Deutsch

Das Hepatitis E-Virus (HEV) kann beim Menschen eine akute oder chronische Leberentzündung hervorrufen. In den letzten zwei Jahrzehnten sind die Zahlen der gemeldeten Hepatitis E-Fälle unter anderem in Deutschland stark angestiegen. Hier spielt vor allem der HEV-Genotyp 3, der auch in Haus- und Wildschweinen weit verbreitet ist, die wichtigste Rolle. Für diesen Genotyp wird als Hauptübertragungsweg der Verzehr von kontaminierten Fleischprodukten aus infizierten Tieren angenommen. Aber auch andere Übertragungswege, beispielsweise über Tierkontakt, kontaminierte Oberflächen oder Blutprodukte, können eine Rolle spielen. Zur Beurteilung des Risikopotenzials spezieller Lebensmittel und Übertragungswege sind Kenntnisse zur Stabilität und Inaktivierung des HEV eine wichtige Voraussetzung, die aber zu Beginn der hier vorgestellten Studien noch weitgehend fehlten.

Ziel der Arbeit war deshalb die Ermittlung grundlegender Daten zur Stabilität von HEV gegenüber verschiedenen physiko-chemischen Einflussfaktoren wie pH-Wert, Salzkonzentration, Trocknung sowie eine Behandlung mit Hochdruck. Hierbei sollten vorrangig Bedingungen untersucht werden, die in der Lebensmittelherstellung zur Anwendung kommen oder nach Kontamination von Oberflächen zu erwarten sind, um praxisnahe Schlussfolgerungen ziehen zu können.

Für die Untersuchungen wurden HEV-Präparationen in Pufferlösungen den entsprechenden Behandlungen unterzogen und anschließend die Restinfektiosität mittels Zellkulturanzüchtung und Immunfluoreszenzanalyse viraler Proteine bestimmt. Nach Optimierung der Methode konnten Virusmengen von etwa 10^4 infektiösen Einheiten pro ml eingesetzt werden, deren vollständige Inaktivierung auf der Basis ähnlicher Vorgaben für die Desinfektionsmitteltestung als ausreichend bewertet wurde.

In der ersten Studie wurde die Stabilität von HEV gegenüber verschiedenen pH-Werten untersucht. Hierbei wurde ermittelt, dass eine effiziente Inaktivierung nach 3 Stunden Inkubation bei Raumtemperatur (RT) nur bei pH 1 und pH 10 erfolgte, während das Virus gegenüber pH 2 - 9 stabil war. Weitere Versuche wurden unter Zusatz von D/L-Milchsäure im Bereich zwischen pH 4,5 und 6,5 durchgeführt, um Bedingungen während der Lebensmittelherstellung und -konservierung zu

simulieren. Hierbei ergab sich nur eine leichte Reduktion der HEV-Infektiosität nach 7 Tagen bei RT, während bei 4 °C unter den gleichen Bedingungen keine signifikante HEV-Infektiositätsreduktion im Vergleich zur jeweiligen Kontrolle bei pH 7,7 feststellbar war.

In der zweiten Studie wurde die Salz-Stabilität von HEV untersucht. Bei Natriumchlorid-Konzentrationen von bis zu 20% konnte nach 24 Stunden bei RT keine Verringerung der HEV-Infektiosität im Vergleich zur Kontrolle festgestellt werden. Auch Salzkonditionen, wie sie bei der Fleischkonservierung Verwendung finden, riefen nach 6 Tagen bei RT bzw. nach 8 Wochen bei 16 °C nur geringfügige Verminderungen der HEV-Infektiosität gegenüber den jeweiligen Kontrollproben ohne Salzzusatz hervor.

In der dritten Studie wurde die Hochdruck-Stabilität von HEV untersucht. Hierbei wurde gezeigt, dass HEV beim höchsten üblicherweise für die Lebensmittelkonservierung verwendeten Druck von 600 MPa für 2 Minuten fast vollständig inaktiviert wurde, während niedrigere Drücke zu geringeren Abnahmen der Infektiosität führten. Längere Druckhaltezeiten und niedrigere Temperaturen führten zu stärkerer Abnahme der HEV-Infektiosität.

In der vierten Studie wurde die Trocknungsstabilität von HEV auf verschiedenen Oberflächen untersucht. Hierbei nahm die Infektiosität von HEV durch den Trocknungsprozess nur wenig ab. Bei der anschließenden Lagerung bei RT und 26% relativer Luftfeuchtigkeit wurde eine vollständige HEV-Inaktivierung erst nach 8 Wochen erreicht. Bei 3 °C und 98% relativer Luftfeuchtigkeit fanden sich nach 8 Wochen auf Plastik noch sehr hohe HEV-Konzentrationen, gefolgt von Keramik und Stahl, während das Virus auf Holz zu diesem Zeitpunkt vollständig inaktiviert war.

Aus den Ergebnissen kann geschlussfolgert werden, dass HEV sehr stabil gegenüber physiko-chemischen Einflussfaktoren ist. Unter Bedingungen, wie sie beispielsweise bei der Rohwurstherstellung vorherrschen, kann HEV nicht effizient inaktiviert werden, weshalb Rohwurstprodukte als potentielle Infektionsquelle angesehen werden müssen. Eine Hochdruck-Behandlung von Risikolebensmitteln könnte eine wirksame Maßnahme zur Erhöhung der Lebensmittelsicherheit darstellen. Da HEV nach Trocknung auf Oberflächen außerordentlich stabil ist, sind strikte Hygienemaßnahmen bei der Lebensmittelzubereitung und in der

Patientenumgebung Voraussetzung für eine Unterbrechung von Infektionsketten. Zusammenfassend geben die ermittelten Stabilitätsdaten grundlegende Einblicke in die Eigenschaften des HEV-Partikels, die für anwendungsorientierte Aspekte in der Lebensmittelproduktion, aber auch für andere Bereiche wie Medizinprodukte oder Krankenhäuser, von Bedeutung sein können.

2 Introduction

2.1 Hepatitis E disease as a global challenge

Infections of humans with the hepatitis E virus (HEV) currently represent a global threat. HEV is the causative agent of human hepatitis E, which is responsible for the most acute hepatitis cases in humans worldwide [1]. In Africa and Asia alone, around 20 million people contract hepatitis E each year [1]. The disease can take an acute or chronic course, depending on the genotype and immune status of each individual patient [2], [3]. Whereas in most cases the course of disease is mild, it can take a dangerous course, mostly in specific risk groups [4]. Especially pregnant women and immunosuppressed patients are affected by severe or even fatal courses, with mortality rates up to 25% in pregnant women [4], [5].

As HEV is prevalent worldwide, the virus can be found in many developing and industrialized countries. However, both the endemic genotypes and their transmission routes differ substantially. Thus, genotypes 1 and 2 are predominant in developing countries, with fecal-oral transmission from person to person via contaminated drinking water or food [6], [7]. An actual example is a long-lasting outbreak in many regions of Namibia over years, which started with an increase of acute jaundice cases in the region of Khomas in September 2017. In December 2017, the Ministry of Health and Social Services of Namibia declared an acute outbreak of hepatitis E in the Khomas region. From there, the virus spread to many other regions, resulting in a national epidemic with a total of 6,151 hepatitis E cases until August 2019 according to the situation report (SITREP No. 64) of the Ministry of Health and Social Services of Namibia [8]. Reasons for this include contaminated drinking water and food, as well as poor sanitation [9] (Fig. 1).

Figure 1. Namibia's Goreangab residents use insufficiently purified water. Figure from web page [10].

In contrast to this, genotypes 3 and 4 are predominantly found in industrialized countries [11]–[14]. These genotypes are zoonotic with pigs and wild boars as the main reservoir animals [15]–[17]. Steeply increasing numbers of hepatitis E cases in several European countries have been reported for the period from 2005 to 2015 [18]. In addition, increasing numbers of notified hepatitis E cases are also evident in Germany for the last two decades, resulting in ~3,000 yearly disease cases in the last years [19]. The main route of transmission from animals to humans is assumed to be the consumption of food from infected animals [16], [20]–[23]. In the last years, reports of meat and meat products contaminated with HEV have also reached the public media in Germany. For example, in August 2021, the German regional television station rbb reported on a random investigation of 10 liver sausage types from various food retailers. According to this, 70% of the liver sausages were contaminated with HEV [24] (Fig. 2).

Figure 2. 70% of liver sausages were contaminated with HEV, according to a random study by the rbb consumer magazine "Super.Markt". Figure from web page [24].

2.2 History of HEV discovery

The first HEV outbreak identified as such in retrospect happened in India in 1955. This outbreak developed into a multi-year epidemic in New Delhi with more than 30,000 cases. Further outbreaks in India followed in the 1970s, e.g. in Ahmedabad or Pune. In these two epidemics, fecally contaminated drinking water could be identified as possible source. But none of the hepatitis-causing viruses known at this time could be found in the studies conducted in 1980. This strengthened the assumption that another human hepatitis-causing virus must exist in addition to the well-known hepatitis A and B viruses [25], [26]. In the following years, more scientific articles on epidemics and sporadic cases of human hepatitis followed, and the agent was called enterically-transmitted non-A/-non-B hepatitis virus [27]–[30]. Through a self-infection, Balayan was able in 1983 to isolate and characterize the novel virus from his feces [27]. Images taken by immune electron microscopy (IEM) showed virus-like particles (VLPs) with diameters from 27 to 30 nm [27]. Purcell and Ticehurst were the first people suggesting the name hepatitis E virus in 1988 [31]. In 1991, Tam et al. succeeded in cloning and sequencing the full-length genome of HEV [32]. The knowledge gained from these studies was crucial for the development of all further HEV-specific molecular biology methods.

In 1997, the first animal HEV strain was discovered in the domestic pig in the USA [33]. The strain, named swine HEV, turned out to be tightly related to, but different from previously known human HEV strains [33]. Soon after, 2 cases of acute hepatitis E in humans were reported in the United States. In both cases, the patients were infected with HEV strains which differed significantly from the known human HEV strains [34], [35], but showed a very close relationship to the discovered swine HEV strain [36]. In the same study, Meng and his colleagues demonstrated experimentally, that the swine HEV strain is able to cross species barriers and infect non-human primates [36].

An indication that HEV-related agents might also occur naturally in other animal species was given in 1999 by Payne et al., who discovered a high genetic similarity between the big liver and spleen disease virus (BLSV, a virus from chicken) and human HEV [37]. In 2001, a similar virus, called avian HEV, was found in the bile of chickens with hepatitis splenomegaly (HS) syndrome [38]. At the same time, more and more animal species containing anti-HEV antibodies were discovered successively in various regions all over the world [39].

In 2014, a new genotype of HEV was discovered in fecal samples of dromedaries from Dubai and the United Arab Emirates [40]. This genotype was classified as the new genotype 7 of HEV *Orthohepevirus A* [41]. Two years later, the first case of a human infection with this genotype was uncovered in the United Arab Emirates, when a liver transplant patient was tested positive for camelid HEV who regularly consumed meat and milk from camels [42].

In 2017, another specific case of hepatitis E due to infection with an unusual HEV strain was described in Hong Kong. A 56-year-old man became infected with rat HEV, which is genetically distinct from HEV-1 to 4 and HEV-7, after a liver transplantation. This rat HEV strain was previously considered as not transmissible to humans [43]. This case clearly shows, that the hepatitis E virus is able to cross barriers between genetically distant host species and can cause previously unsuspected zoonotic infections.

2.3 HEV taxonomy and classification

The hepatitis E virus is actually classified into the *Hepeviridae* family according to the International Committee on Taxonomy of Viruses (ICTV) [44]. The current classification is schematically shown in Figure 3. The family is divided into two genera: The genus *Piscihepevirus*, which currently contains HEV-related viruses of aquatic vertebrates integrated into the sole species *Piscihepevirus A*, and the genus *Orthohepevirus* for those of terrestrial vertebrates. The latter genus contains the four species *Orthohepevirus A* to *D*. The species *Orthohepevirus B* includes HEV-related viruses detected in birds (avian HEV) with genotypes I-IV, *Orthohepevirus C* includes HEV-related viruses from rodents (genotype C1) and carnivores (genotype C2), whereas HEV-related viruses from bats are included in the species *Orthohepevirus D* (bat HEV). The species *Orthohepevirus A*, also known as HEV in a stricter sense, comprises eight genotypes, of which types 1 to 4 and 7 have been shown to be pathogenic to humans. Whereas both genotypes 1 and 2 circulate exclusively in humans, the other three human-pathogenic genotypes with zoonotic potential circulate in specific host animals. A phylogenetic investigation revealed that the division into anthropotropic (HEV-1 and HEV-2) and zoonotic genotypes (HEV-3 and HEV-4) took place about 536 to 1,344 years ago [45]. The eight genotypes can be further divided into 36 subgenotypes, based on genome sequence identities [41], [46]–[48].

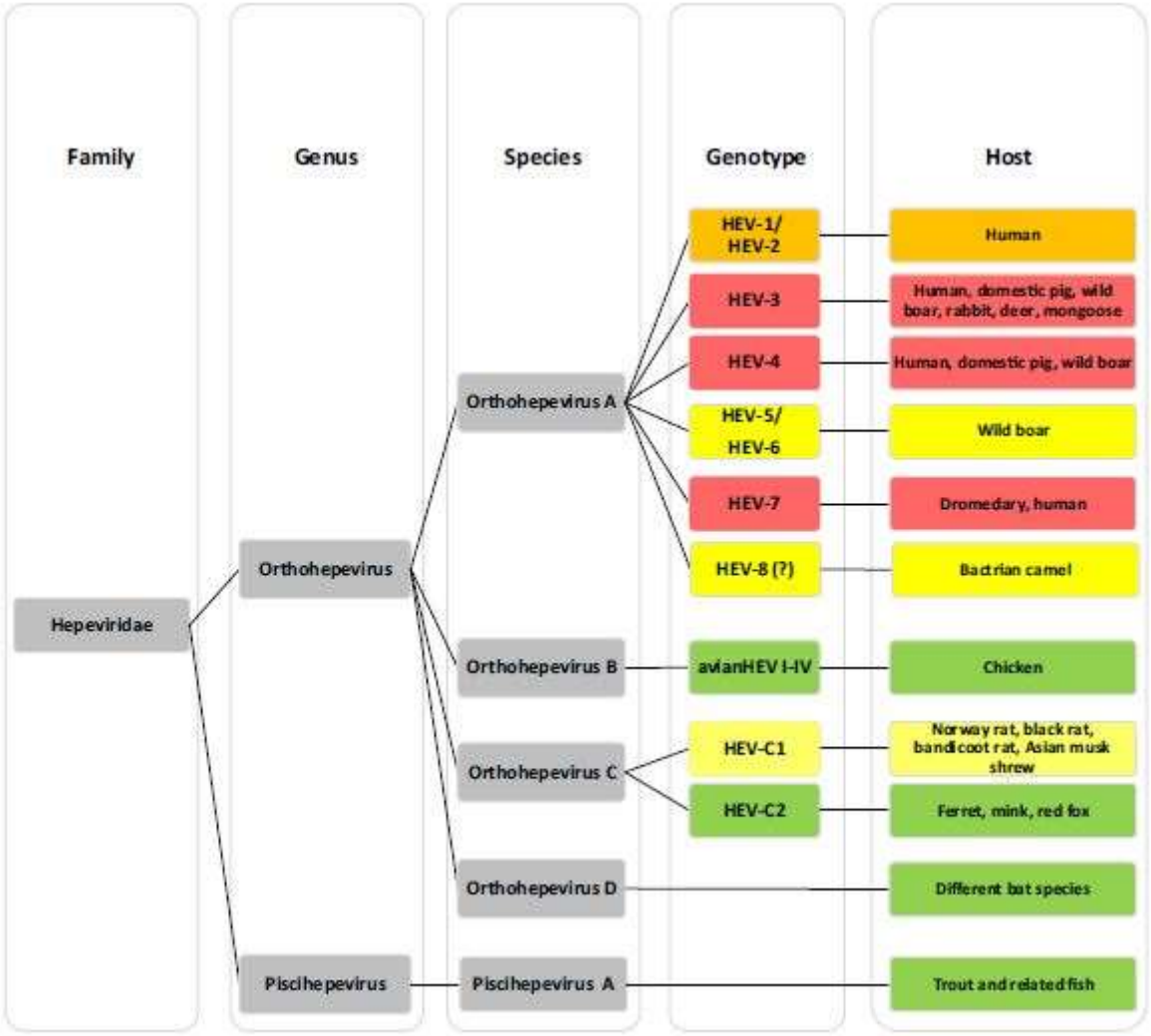

Figure 3. Taxonomical classification of HEV and HEV-related viruses based on whole-genome sequences according to [41]. Colors indicate the zoonotic potential of the genotypes: orange (human-to-human transmission only), red (animal-human transmission proven), yellow (animal-human transmission not proven, but maybe possible due to relationship to human strains or serological evidence) and green (no animal-human transmission expected). Figure from journal article [48].

2.4 HEV genome and virus structure

HEV is characterized by a small virus particle containing a single-stranded ribonucleic acid (RNA) genome with a positive polarity [44]. The genome is about 7.2 kilobases large and is composed of a short 5'-non-coding region capped with 7-methyl-guanosine, three open reading frames (ORFs) and a short 3'-non-coding region polyadenylated at the end [49] (Fig. 4).

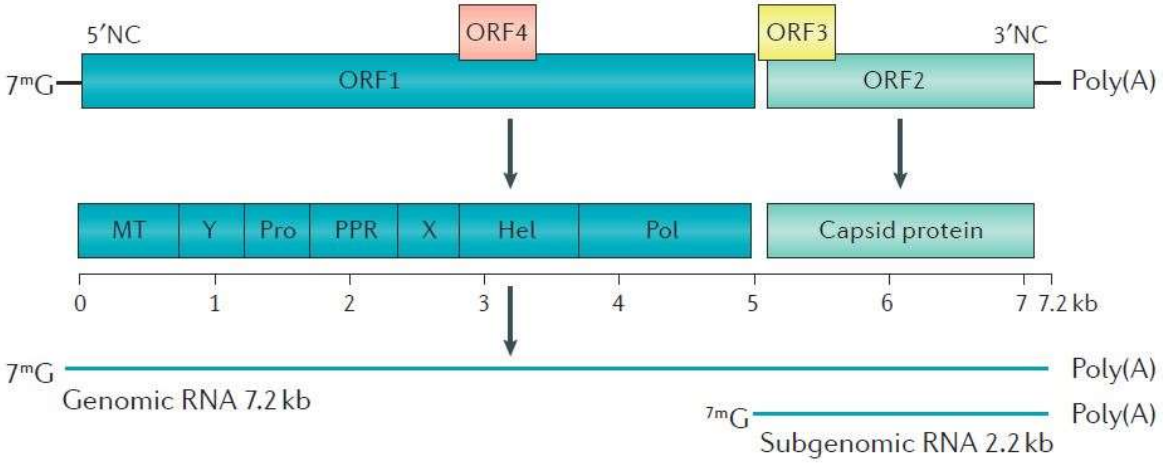

Figure 4. HEV genome showing its ORFs (top), the generated proteins (middle) and the types of viral RNA (bottom). ORF1 encodes a polyprotein containing domains for methyltransferase (MT), region Y (Y), cysteine protease (Pro), polyproline region (PPR), region X macro (X), RNA helicase (Hel) and RNA polymerase (Pol). Figure from journal article [49].

ORF1 encodes a polyprotein containing domains, which are essential for RNA replication, including a methyltransferase , a cysteine protease , an RNA helicase and an RNA polymerase , as well as three regions with unknown function (Y domain, polyproline region and X macro domain) [50]. ORF2 encodes the capsid protein, which is organized in three domains: the shell, the middle and the protruding domain [51]. The latter possesses the most epitopes as targets for neutralizing antibodies and probably a receptor binding domain [51], [52]. Ten capsid proteins accumulate to form a decamer, and 18 decamers form the capsid icosahedron, which encapsulates the viral genome [53] (Fig. 5).

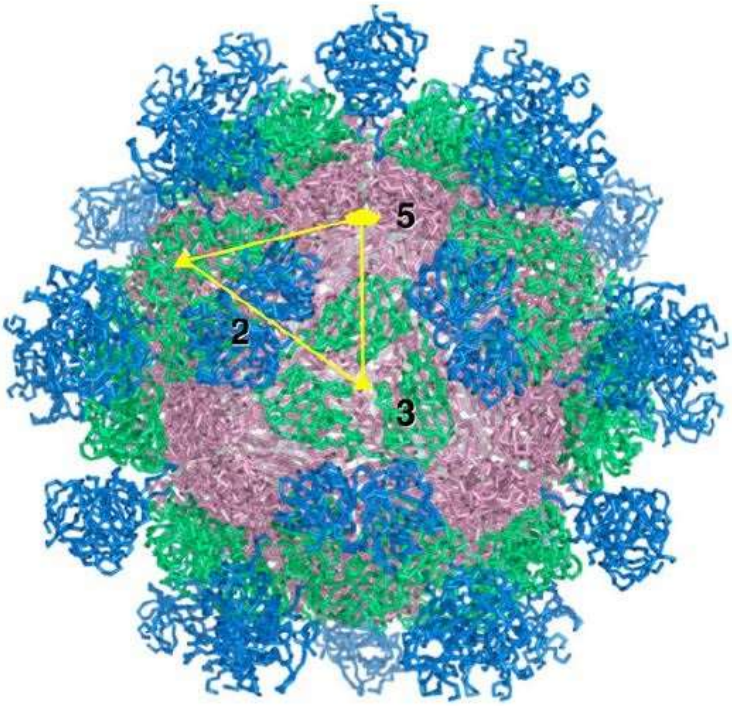

Figure 5. Crystal structure of the non-enveloped form of the HEV capsid. Shell domain = pink, middle domain = green and protruding domain = blue. Figure from journal article [51].

ORF3 encodes the small ORF3 protein, which is mandatory for virus release [49]. Additionally, HEV-1 possesses another ORF, the ORF4. ORF4 encodes a protein, which is expressed upon endoplasmic reticulum (ER) stress and leads to increased viral polymerase activity [54].

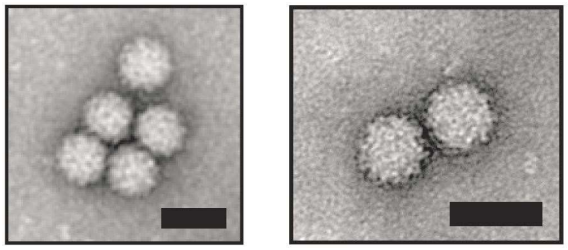

Figure 6. Transmission electron microscopic images of quasi-enveloped HEV particles from purified cell culture supernatant of HEV-infected cells. Bars: 50 nm. Figure from journal article [55].

Morphologically, the HEV capsid strongly resembles that of caliciviruses; therefore, it was previously taxonomically classified in this family [56] (Fig. 6). However, it is now evident, that two different particle types of HEV exist: non-enveloped (naked) and

quasi-enveloped (cloaked) HEV particles [49]. Compared to the non-enveloped particle, the quasi-enveloped particle additionally contains a lipid membrane and ORF3 proteins [49]. Whereas non-enveloped particles have a diameter of about 30 nm, quasi-enveloped particles are about 40 nm in diameter [32], [55]. The density for non-enveloped particles is 1.25 g/ml on average, whereas the density for quasi-enveloped particles is 1.15 g/ml on average [49], [55], [57]–[59]. Whereas non-enveloped particles could be detected mostly in stool, quasi-enveloped particles were found as predominant particle type in blood and cell culture supernatant [58], [60]. Although both particle types are infectious in cell culture, the non-enveloped form shows higher infectivity [61], [62]. Protection against neutralizing antibodies has been demonstrated for the quasi-enveloped particles due to their lipid membrane, which may result in an immune evasion of HEV in the host organism [63].

2.5 HEV replication and pathophysiology

In most cases, HEV infection is started by entering the gastrointestinal (GI) tract of humans through the oral cavity. Although the exact mechanism of HEV entry has not yet been completely elucidated, uptake by infection of intestinal cells seems very likely [64]. In line with this, tissues of the GI tract have been shown to serve as extrahepatic replication sites of HEV [65], [66]. Thereafter, the virus enters the liver via the portal vein blood, which in the end results in liver inflammation [60]. For liver cell infection, the virus particle binds to heparan sulfate of the corresponding glycoprotein from the host cell membrane, followed by endocytosis by the clathrin-mediated transport system under involvement of a so far unknown receptor [49]. Virus uncoating takes place in the cytosol [49]. Thereafter, ORF1 is translated from released viral RNA into the non-structural proteins, including the viral RNA polymerase [49]. This generates a negative-sense transcript of the whole viral genome, which acts as template for positive-sense full-length genome copies as well as a subgenomic messenger-RNA (mRNA) [49]. The subgenomic mRNA is translated into the capsid protein (from ORF2) and a phosphoprotein (from ORF3) [49]. After the capsid protein has crossed the ER, it forms new virus particles with lipid membranes, together with the ORF3 protein and positive-sense viral genome [49]. Since HEV replication does not cause a cytopathic effect, the clinical picture of liver damage cannot be explained by the virus replication itself. Rather, an immune

response mediated by cytotoxic T cells and natural killer cells is assumed to be responsible for inflammation and destruction of infected liver cells [64]. The release of the assembled virus particles proceeds via the exosomal pathway (budding) with participation of the ORF3 protein, resulting in excretion of quasi-enveloped particles [49] (Fig. 7). Therefore, HEV particles released into the blood mainly contain lipid membranes, although non-enveloped particles have also been detected in serum of patients [60], [67]. In contrast, HEV particles released into the biliary capillaries lose their lipid membranes and ORF3 proteins due to the emulsifying effect of bile acid salts in the bile fluid [49]. These non-enveloped viruses enter the GI tract via the bile duct and are shedded with the feces into the environment [49], [68]. Other tissues in which HEV has been shown to replicate include kidney, central nervous system and placenta [69]–[71].

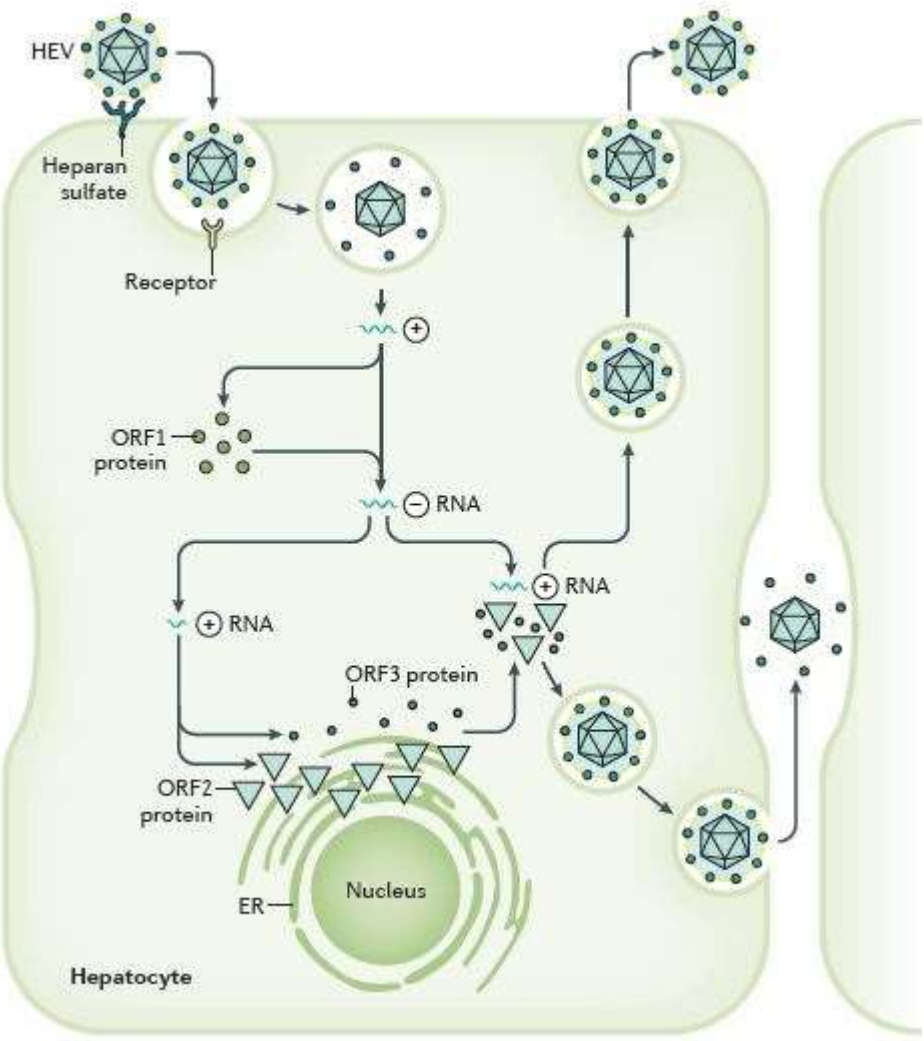

Figure 7. HEV replication in hepatocytes. Figure from journal article [49].

2.6 Hepatitis E disease and treatment options

Acute and chronic disease courses of hepatitis E have been described in humans. A typical acute course of disease occurs in 5 to 30% of people infected with HEV, and begins with an incubation period of about 2 to 8 weeks, with 40 days on average [72]. Initial unspecific symptoms such as malaise, fever, anorexia, nausea, emesis and abdominal pain occur in a short prodromal phase, which can last up to a week. This is followed by the icteric phase, which is characterized by dark-colored urine and jaundice [49]. A physician might palpate hepatomegaly from then on [72], [73]. Jaundice and its associated symptoms mostly disappear after a few days to several weeks. With disappearance of symptoms, the convalescent phase begins, which is characterized by significant improvement of the patient's general condition [49].

Despite the typical icterus, a clinical diagnosis for hepatitis E is very difficult, because other viral hepatitis diseases also present with this symptom [74]. Thus, a differential diagnosis is necessary, which needs a laboratory analysis. This is performed either directly by determining HEV RNA or capsid antigen in blood or other body fluids, or indirectly by determining anti-HEV antibodies in blood [75]–[77]. The RNA of HEV can be found in the blood and feces during the incubation period. It persists about 4 weeks in the blood and about 6 weeks in feces. Capsid antigen persists also about 4 weeks in the blood [78]. After 2 to 6 weeks of incubation period, short-living immunoglobulin M (IgM) antibodies become detectable, increase and remain up to 9 months [79]. The long-living immunoglobulin G (IgG) immune response can occur temporally delayed, but then remains for a few years [49]. Around the same time of the increase in IgM antibodies, elevated levels of liver enzymes as alanine aminotransferase (ALT, an important marker of liver inflammation or injury) can also be measured [79] (Fig. 8). Other marker for a HEV-infected liver are: γ-glutamyltransferase and bilirubin. High concentrations of bilirubin can be measured during the icteric phase [80]. Further indicators are the already mentioned dark urine, and decolorized feces [73].

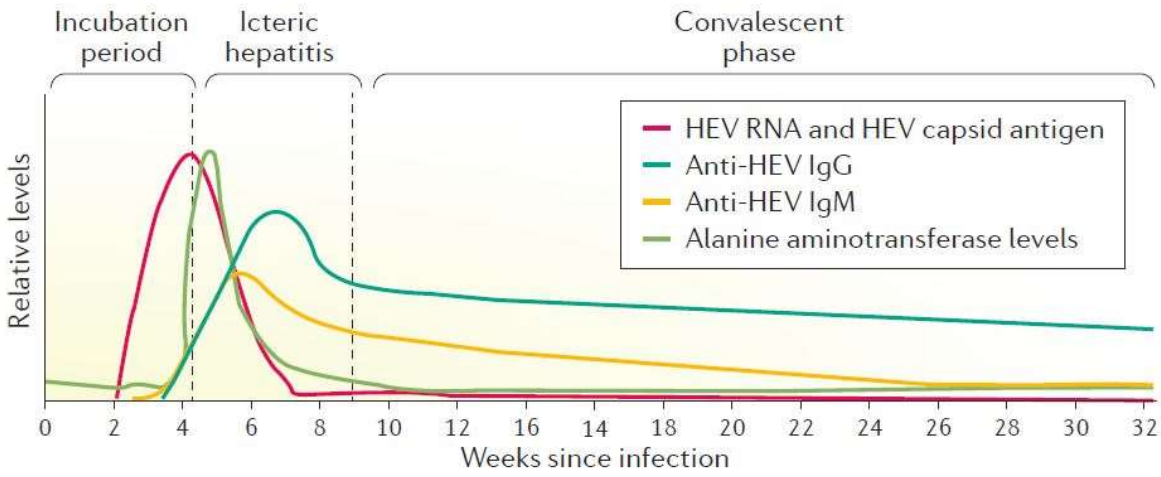

Figure 8. Time-course of HEV infection and disease in humans. Curves of HEV RNA, capsid protein and IgM & IgG antibodies, as well as alanine aminotransferase levels. Figure from journal article [49].

Immunocompetent patients normally recover from acute hepatitis E without the need of antiviral medication [49]. However, 0.5 to 4% of people with acute hepatitis E progress to life-threatening acute liver failure [81]. Preexisting chronic liver diseases can increase the risk of acute liver failure up to a fatality rate of 67% [82].

The course of an acute hepatitis E disease also depends on the involved HEV genotype. Infections with HEV-1 and HEV-2 range from completely asymptomatic cases up to fulminant cases with acute liver failure [49]. For HEV-1 infection, pregnant women constitute a special risk group. Especially in the second and third trimesters, there is a high risk of developing acute icteric hepatitis E. A high percentage of these women also develop acute liver failure. Thus, the fatality rate of pregnant women is between 15 and 25% [5]. But also the risk for premature births, miscarriages and stillbirths increases, and the newborns can contract the virus from their mothers [83]. Nevertheless, the reason for this high fatality rate among pregnant women is still unclear and needs to be elucidated [84]. In contrast, infections with HEV-3 and HEV-4 usually present milder clinical symptoms [49]. Fulminant courses with acute liver failure are rare after infection with these genotypes. Nevertheless, single cases have been reported in Europe [85]–[88]. Also, pregnant women do not represent a special risk group here [89], [90].

However, in case of infection with HEV-3 or HEV-4, infection can persist for more than 3 to 6 months resulting in chronic hepatitis E with proceeding liver fibrosis and

cirrhosis [91]. This form has been frequently described in adults and children, who obtained immunosuppressive therapy due to solid organ transplantation (SOT) or stem cell transplantation, in patients with chemotherapy or immunotherapy and in patients with concomitant human immunodeficiency virus (HIV) infection [92]–[100]. Among chronic HEV infections, genotype 3 represents the majority, whereas genotype 4 plays only a minor role [101]. Until now, HEV-1 and HEV-2 have not been detected in chronic hepatitis E patients [102]. Chronic hepatitis E courses most commonly occur in patients with SOT [94]. Approximately one third of these patients eliminate the virus spontaneously, whereas approximately two thirds develop chronic hepatitis E [94], [103]. Thereafter, disease progresses to cirrhosis with a rate of about 10% [94], [104]. However, most of the immunosuppressed patients infected with HEV have an asymptomatic course [94]. Furthermore, laboratory diagnosis of hepatitis E is also more difficult in this patient group, as false-negative IgM and IgG tests are frequent due to immunosuppression [94].

Besides hepatic disease, several extrahepatic manifestations of hepatitis E virus infections have been suggested, although with different degrees of evidence. Most data are available for neurological and renal manifestations [49]. Neurological manifestations have been described in immunocompetent patients, as well as in immunocompromised patients with chronic courses due to HEV-3 infection [49]. The best investigated neurological manifestations are Guillain-Barré syndrome, encephalitis, myelitis and neuralgic amyotrophy [105]. These observations are supported by studies, showing that HEV is able to replicate in several neurological cell lines (*in vitro*) and is able to overcome the blood-brain barrier (*in vivo*) [70], [106], [107]. Also, renal manifestations have been found in patients with acute, as well as chronic hepatitis E [108], [109]. Renal biopsy samples, taken from patients infected with HEV-1 or HEV-3, indicate glomerular clinical pictures, including membranoproliferative glomerulonephritis with or without cryoglobulinemia and membranous glomerulonephritis [109]–[112]. A clearance of HEV is related with a decline of cryoglobulinemia, an improvement of kidney function based on estimated glomerular filtration rate and a decrease of proteinuria [109], [112], [113].

In contrast to humans, the course of hepatitis E is quite different in animals. Infections with HEV and its related viruses occur in various animal species, including farm animals, pets, wild animals, laboratory animals and zoo animals [48]. In general,

infections with these viruses seem to proceed here without any clinical symptoms or pathological-histological modifications. This is also the case in HEV-infected pigs and wild boars. Only chickens naturally infected with avian HEV and primates (cynomolgus macaques) experimentally infected with HEV-1 show clinical symptoms [37], [38], [48], [114], [115]. During an acute infection with avian HEV, chickens can present clinical symptoms and pathological-histological modifications such as hepatomegaly, splenomegaly, modifications of kidney, microsomia, decrease in egg laying capacity, accumulation of body fluids in the abdomen, hepatic vasculitis and amyloidosis as well as an increased fatality rate [114], [116].

The options for pharmacotherapy of hepatitis E differ significantly depending on the disease course and the patient's immune status. In the case of acute disease in immunocompetent patients, pharmacotherapy is mostly not needed because HEV infection is cleared spontaneously and accompanied by recovery of disease [49]. For fulminant hepatitis courses, antiviral therapy with ribavirin - a nucleoside analogue acting against several DNA and RNA viruses - could be considered. Rapid healing under ribavirin therapy has been reported for small groups of patients with acute hepatitis E in several recent studies [117]–[120]. Corticosteroids represent another therapeutic option. These could reduce the risk of acute liver failure in patients with fulminant hepatitis E [49], and improvement of liver function under corticosteroid therapy has been reported in single cases [87].

In the case of immunosuppressed patients, intensive physicians' care including continued adjustment of the respective pharmacotherapy is often required in order to prevent chronification of hepatitis E. If possible, immunosuppression should be reduced for 3 months, during which time the HEV RNA load should be monitored monthly [49]. If HEV RNA persists in serum or stool, 3 months of monotherapy with ribavirin should be initiated [49]. In case of relapse after ceasing the drug, a 6-month treatment is continued under the same conditions [49]. However, the therapy may not be successful because of the occurrence of ribavirin-resistant HEV strains or the appearance of severe side effects due to ribavirin treatment [121]. In case of further HEV RNA persistence in serum or non-response to therapy, follow-up therapy with pegylated interferon-alpha for 3 months can be considered [49], [121]. However, this therapy is also not without risk, as it stimulates the immune system and thus increases the risk of acute rejection in patients with SOT [122]. Therefore, it is only

suggested for patients with a transplanted liver, whereas it is not recommended for all other transplanted organs [49].

2.7 HEV epidemiology

Today, hepatitis E virus is considered as endemic in various regions worldwide. Nevertheless, a certain regional distribution can be attributed to the most important human pathogenic genotypes (Fig. 9).

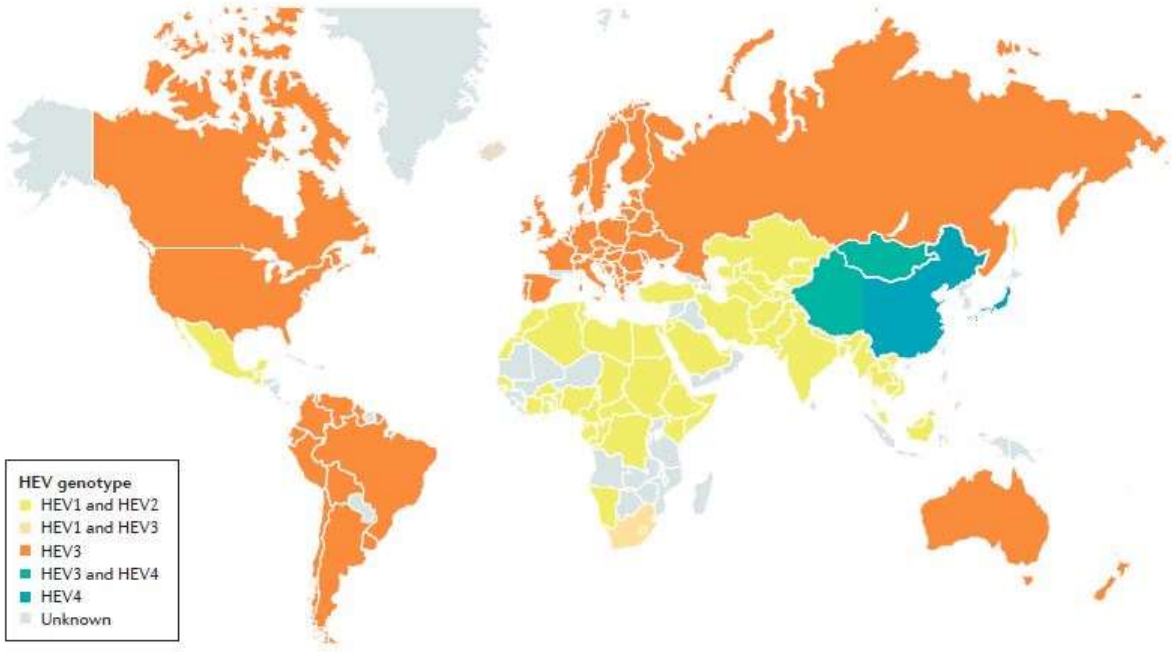

Figure 9. Scheme of the global distribution of different HEV genotypes. Figure from journal article [49].

HEV-1 is predominant in Mexico, Africa, the Middle East and several regions of Asia as central, south and southeast Asia, whereas HEV-2 is detected only in Mexico and Africa [49]. Mathematical models from 2012 demonstrate a huge impact of HEV-1 and HEV-2, leading to an approximated incidence of 20.1 million people per year in Africa and Asia with 3.4 million cases of acute hepatitis E, 70,000 deaths owing to acute liver failure and 3,000 stillbirths [1]. Outbreaks of HEV-1 and HEV-2 can differ strongly in the number of affected people, from little groups to several thousand people with disease attack rates up to 15% [123]–[125]. Seroprevalences of anti-HEV antibodies range from 10 to 40% in many regions of Africa and Asia. The highest

seroprevalences are found in groups with age over 50 years. Reported seroprevalences of anti-HEV antibodies in Egypt are exceptionally high at around 80% [126], [127].

HEV-3 is predominant in most other regions worldwide, whereas HEV-4 is almost exclusively found in China, Japan and Mongolia [49]. In this context, China is exemplary for a newly industrialized country. Improvements in sanitation facilities substantially reduced outbreaks of HEV-1 in the last few decades, whereas more and more sporadic cases of HEV-4 infections are being recorded [49], [128].

In Europe, the seroprevalence depends strongly on the investigated regions. The highest seroprevalences are found in Southwest of France with rates over 50% [129]. Seropositivities of 10 to 30% are found in Northern France, the United Kingdom, Belgium, the Netherlands, Luxembourg and Germany [130]. Smaller seroprevalences of less than 5% were found in adults in Scotland [129]. Seroprevalences increase with advancing age; symptomatic cases of hepatitis E are found primarily in men over 50 years [131].

Between 2013 and 2015, hepatitis E cases increased to be the most diagnosed acute viral hepatitis in the Netherlands [132]. A study from 30 European countries demonstrated an increase from 514 to 5,617 hepatitis E cases per year in the period of 2005 to 2015. Most of them were aquired locally [18]. The situation in the United States is more complex due to a disheveling infrequency of notified hepatitis E cases [133]. Recently revised studies show a prevalence of anti-HEV IgG antibodies of 6% detected in blood donors and in the general population [134], [135]. Infections with HEV-1 in Europe and the United States are very rare, and in all cases associated with a travel [136].

Also in Germany, HEV is considered as an endemic pathogen. Since 2001, infections with HEV are notifiable. Since that time, the Robert Koch Institute (RKI) has observed steeply increasing hepatitis E case numbers from year to year. Only in 2020, a slight decline was recorded, which may be attributed to the COVID-19 pandemic situation (Fig. 10). Most of the cases notified in 2020 were autochthonous (95%); travel-associated cases were a minority. The highest prevalence was observed in men between 50 and 79 years of age [19].

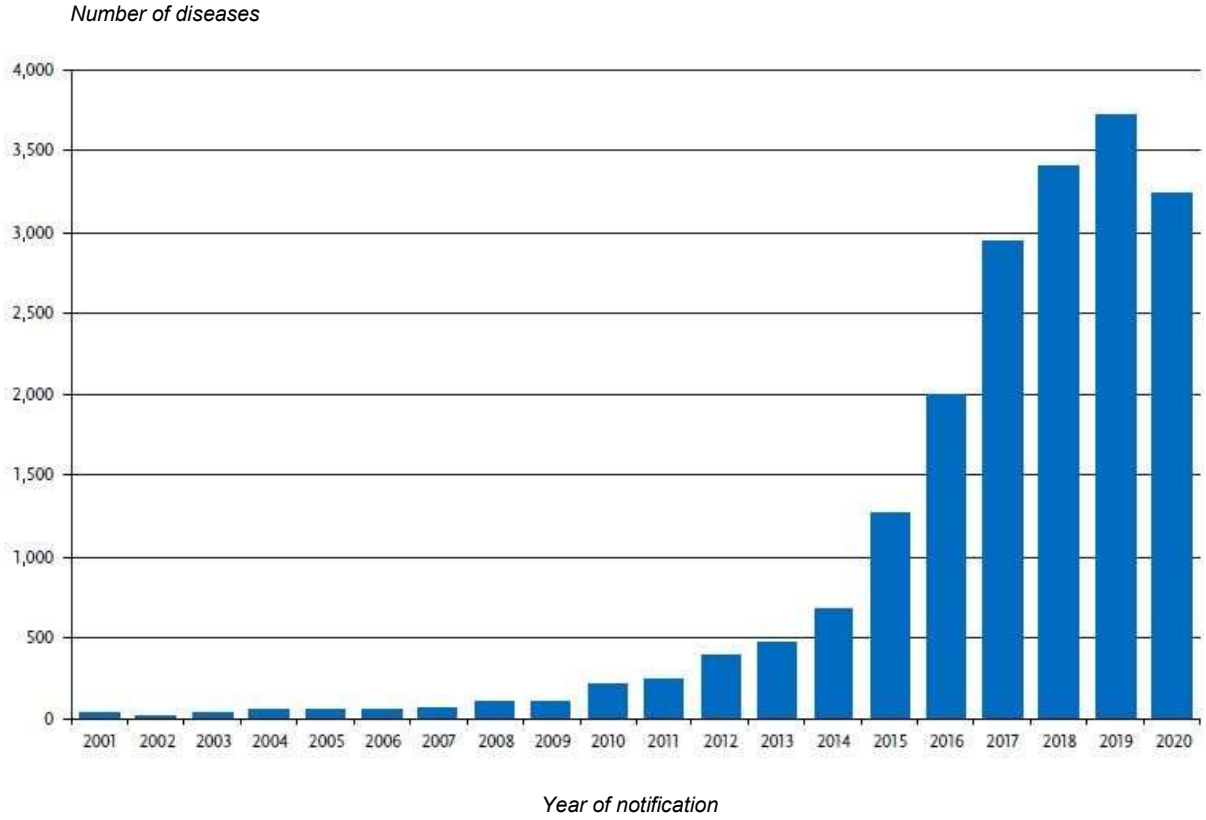

Figure 10. Communicated hepatitis E infections by year of notification, Germany, 2001 to 2020. Figure from report [19].

2.8 HEV transmission pathways

The HEV transmission pathways are very complex and related to the specific genotypes [48]. In general, the virus usually infects humans through use of fecally contaminated drinking water or consumption of meat or meat products from infected animals [49]. But also other transmission routes are reported, such as direct contact to infected animals, humans or contaminated environment, as well as via contaminated blood or blood products (Fig. 11). However, some of them are well proven, whereas others are only suspected [48].

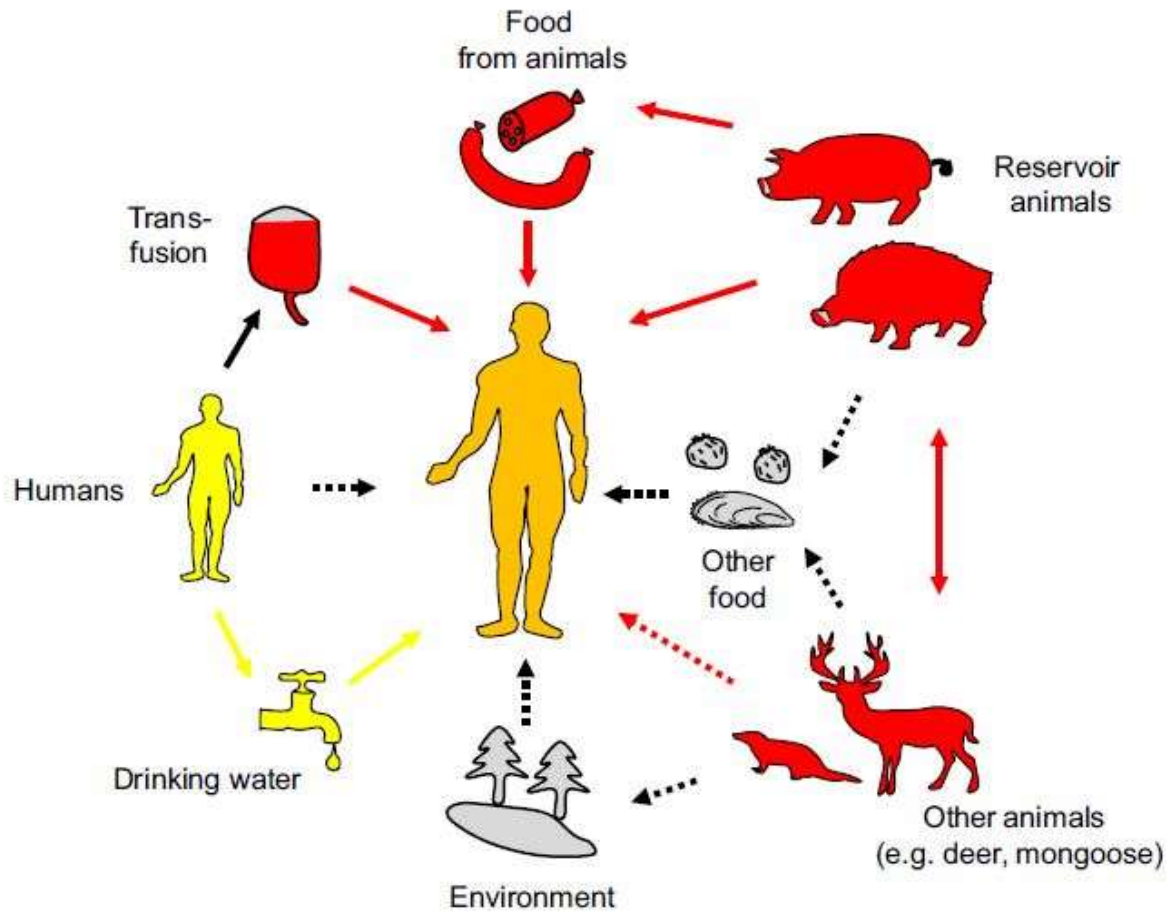

Figure 11. Transmission routes of human pathogenic HEV genotypes. Transmissions, which are well proven, are indicated by bold arrows. Dotted arrows are used, if transmissions are rarely shown or only speculated. Yellow: main transmission routes for genotypes 1 and 2. Red: main transmission routes for genotypes 3 and 4. Figure from journal article [48].

HEV-1 and HEV-2 are restricted to humans under natural conditions. The main transmission route of these genotypes is via fecally contaminated drinking water or food. Limited access to clean water and low hygienic standards have therefore led to many outbreaks in developing regions of the world in the past. During these outbreaks, direct contact from person to person as well as sharing of utensils for eating and drinking in households may additionally contribute to spread of the virus [48].

HEV-3 and HEV-4 primarily circulate in distinct reservoir animals. From there, the virus is transmitted zoonotically to humans by different routes. In this context, domestic pigs and wild boars represent the most important reservoir animals [137], [138]. In addition, several HEV-3 transmissions from deer to humans have been described, although this animal species itself seems to be mainly infected by contact

to wild boars [139]. A distinct subtype (HEV-3ra) has been repeatedly detected in rabbits, which can also serve as food. The same subtype was found in a few human patients [65], [140]–[142].

The consumption of products from infected animals is considered to be most important [48]. Especially undercooked meat and raw sausages produced from infected pigs, wild boars or deer have been often described as sources of human infections with HEV-3 and HEV-4 [143]–[146]. A study indicated, that viral RNA of HEV-4 can be excreted by cow milk [147]. Other food types, such as shellfish and berries, may also act as vehicles for virus transmission after environmental contamination with feces, although this has been shown only rarely [148]–[152].

Transmission of HEV-3 and HEV-4 to humans due to direct contact with infected animals has been often suggested. Many studies showed, that professionals working with animals, such as veterinarians, pig farmers and slaughterers, show significant higher anti-HEV antibody prevalences than the general population [153], [154]. The same phenomenon can be observed in persons, who frequently spend time in nature and thus have contact with infected wild animals, such as wild boars, as well as with their excreta. These include foresters, lumberjacks and hunters [155], [156].

Another important transmission pathway for HEV-3 to humans is iatrogenic via contaminated blood, blood products or organs [92], [157], [158]. As an example, HEV RNA was found in one of 679 to one of 4,252 blood donations in Germany [159].

HEV-7 is widely distributed in Middle Eastern dromedary camels and zoonotic transmission to a human patient has been shown, although the distinct transmission pathway is not known [40], [160]. For HEV-8 detected in farmed bactrian camels from China as well as for HEV-5 and HEV-6 found in wild boars from Japan, no transmissions to humans are known so far [161], [162]. In contrast, members of the species *Orthohepevirus C* seem to possess a zoonotic potential to humans. Two serological studies from German forestry workers and febrile Vietnamese patients demonstrated a higher reactivity of anti-HEV antibodies with rat HEV (HEV-C1) than with HEV-1 or HEV-3 [155], [163]. Recently, several hepatitis E cases in Hong Kong were identified to be caused by rat HEV, although the distinct transmission pathway of the virus could not be clarified [43]. For all other HEV-related viruses, there is so far no evidence for a zoonotic transmission to humans [164]–[166].

2.9 Detection of HEV-3 in animals and food

In Germany and many other European countries, human HEV infections are mainly caused by genotype 3 of subtypes c, e and f [167]. These subtypes are also detected in pigs, wild boars and other animal species [168]. Worldwide, numerous studies are available on the prevalence of HEV-3 in pigs [15]. However, studies of anti-HEV antibody prevalence must be distinguished from those of HEV RNA prevalence. Whereas antibodies in most cases indicate a previous infection and thus can provide data on infections that occurred a long time ago, RNA detections indicate an infection at the time of examination [168]. Also for Germany, data from numerous studies on HEV-3 prevalence in pigs and wild boars are available (Tab. 1).

Animal species	**Detection of HEV RNA Number of positive/total number of tested animals (%)**	**Detection of HEV-specific antibodies Number of positive/total number of tested animals (%)**	**Reference**
Pig	-	534/1072 (49.8%)	[169]
	-	354/516 (68.6%)	[170]
	34/251 (13.5%)	-	[171]
	-	1065/2273 (46.9%)	[172]
	-	187/438 (42.7%)	[173]
	1/105 (1.0%)	-	[174]
	3/120 (2.5%)	-	[175]
	43/259 (17.2%)	155/250 (62.0%)	[176]
Wild boar	17/189 (5.3%)	-	[177]
	22/148 (14.9%)	-	[178]
	48/126 (38.1%)	32/107 (29.9%)	[179]
	-	109/330 (33.0%)	[180]
	18/124 (14.5%)	-	[174]
	14/134 (10.4%)	-	[181]
	39/232 (16.8%)	81/180 (45.0%)	[139]
	4/104 (3.8%)	12/104 (11.5%)	[182]

Table 1. Frequency of HEV infections in pigs and wild boars in Germany. No data are available for cells with the sign - . Table from journal article [168].

According to these studies, at the time of slaughter, antibody prevalences in pigs ranged from 42.7 to 68.6%, whereas RNA prevalences ranged from 1.0 to 17.2%. This reflects that HEV infections in pigs occur mainly in the first months of life, whereas later on the virus has often already been eliminated from the body [168]. Furthermore, slaughtering of piglets is rather rare compared to slaughtering of adult animals. For wild boars, antibody prevalences of 11.5 to 45.0% and RNA prevalences of 3.8 to 38.1% have been reported. It is noticeable, that these values are more scattered among the wild boars than among the pigs. One explanation might be that large differences can be present in infection rates of wild boars from the different investigated geographical areas [156], [178]. In addition, a more heterogeneous age structure of the hunted wild boars and the observation that older wild boars carry HEV RNA more frequently than older pigs may explain these differences [168].

There is some indication that also other animal species can carry HEV-3 [48]. For example, rabbits show high detection rates, which are mainly attributed to a specific rabbit subtype (HEV-3ra). Since sporadic human cases with this subtype have already described, a risk of infections by contact to rabbits has to be considered [168]. HEV RNA was also detected in small wild ruminants. A German study assessed HEV RNA prevalences of 6.4% for roe deer and 2.4% for red deer [139]. Since positive detections in wild ruminants occurred mainly in the presence of simultaneously high HEV prevalences in wild boars of the same area and the detected strains were often identical, spillover infections are assumed in this context [168]. In contrast, a study focusing on domestic cattle yielded only negative results [183]. For other animal species in Germany, only few data are available. One study, in which zoo animals were analyzed, found high antibody prevalences especially in porcine species [184].

As already mentioned, the consumption of meat and meat products from infected pigs and wild boars plays a major role in HEV-3 transmission to humans. Several studies have addressed the question, if HEV RNA can be detected in such food in Germany (Tab. 2).

Food	Detection of HEV RNA Number of positive/total number of tested samples (%)	Reference
Liver	8/200 (4.0%)	[185]
	2/41 (4.9%)	[186]
Liver sausage	11/50 (22.0%)	[187]
	5/40 (12.5%)	[186]
Liver pâté	6/40 (15.0%)	[186]
Raw sausage	14/70 (20.0%)	[187]
without liver content	0/10 (0.0%)	[186]

Table 2. Detection of HEV RNA in food containing pig liver or meat, which was purchased in Germany. Table from journal article [168].

Among the tested pig livers, HEV RNA detection rates of 4.0 to 4.9% were found. Among the tested liver sausages, the detection rates ranged from 12.5 to 22.0%. The detection rate in liver pâté was 15.0%. Detection rates of 0.0 to 20.0% were found for the tested raw sausages without liver content. The use of different methods with different detection limits and the sometimes large differences in sample sizes may explain the differences between studies within a group of foods [168]. In general, the higher detection rates for sausages compared to the starting material liver seem surprising at first. However, it must be considered that during the manufacturing process of a particular sausage product, organs and meat from several animals are mixed, portioned and filled, which can explain the higher detection rates [168]. Interpretation of the data with regard to potential infectivity of the food products is generally problematic, because only RNA was analyzed in these studies. Even currently, only polymerase chain reaction (PCR)-based protocols are available for HEV detection in complex matrices such as food, which cannot distinguish between infectious and inactivated virus.

2.10 Pathogen inactivation during food production

Foods are based on organic substances originating from plants or animals, which are consumed by humans and animals, in order to maintain their metabolism. In addition to their main components such as carbohydrates, proteins and lipids, they contain other organic substances, as well as minerals and water [188]. Thus, they are subject to spoilage due to physical, chemical and microbial processes (Fig. 12), thereby changing nutritional values, color, texture, odor, taste and edibility [189]. In the worst case, foods become inedible, toxic or can cause infectious diseases [190]. In order to prevent spoilage and enable longer storage, it is required to preserve foods [188]. The history of food preservation dates back to ancient civilization, when the primitive troupe first felt the necessity for preserving food after hunting a big animal, which could not be immediately consumed in whole [188]. The knowledge of these techniques was essential to establish civilization [188].

The mechanisms of food spoilage can be grouped into physical, chemical or microbial ones. Figure 12 gives an overview on the mechanisms and their different characteristics.

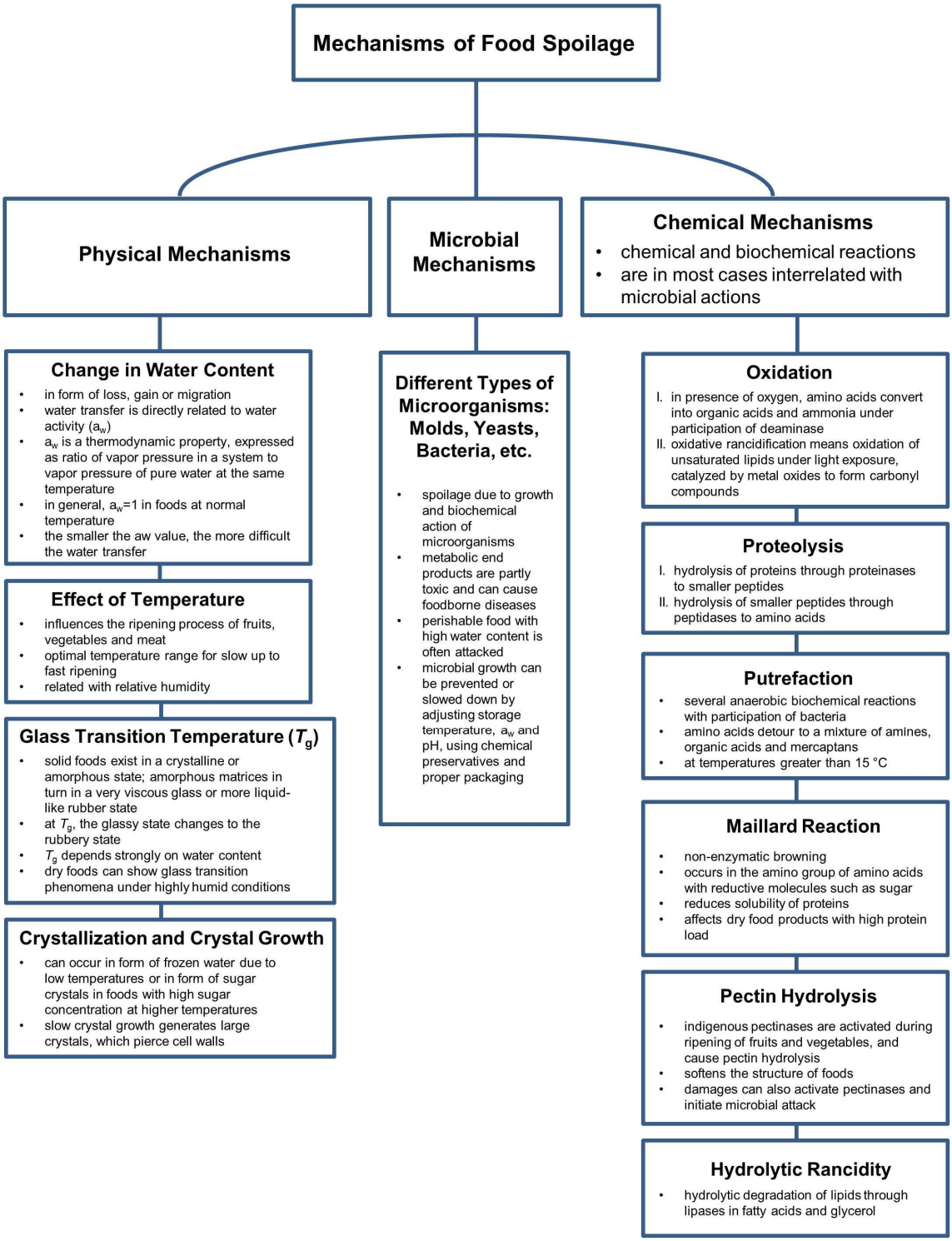

Figure 12. Mechanisms of food spoilage. Modified Figure from journal article [188].

A wide variety of methods for food preservation is known. Whereas some of them can only extend the shelf life of foods, others can also inactivate pathogens possibly contained in foods [188]. Thus, the specific design of food manufacturing processes can have an important impact on food safety. The various methods of food preservation can be divided into three main categories: chemical, biological and physical processing [188]. Figure 13 gives an overview on the different methods. In most cases, combinations of different processing methods are applied to distinct food types, thereby increasing the preservation efficiency and food safety.

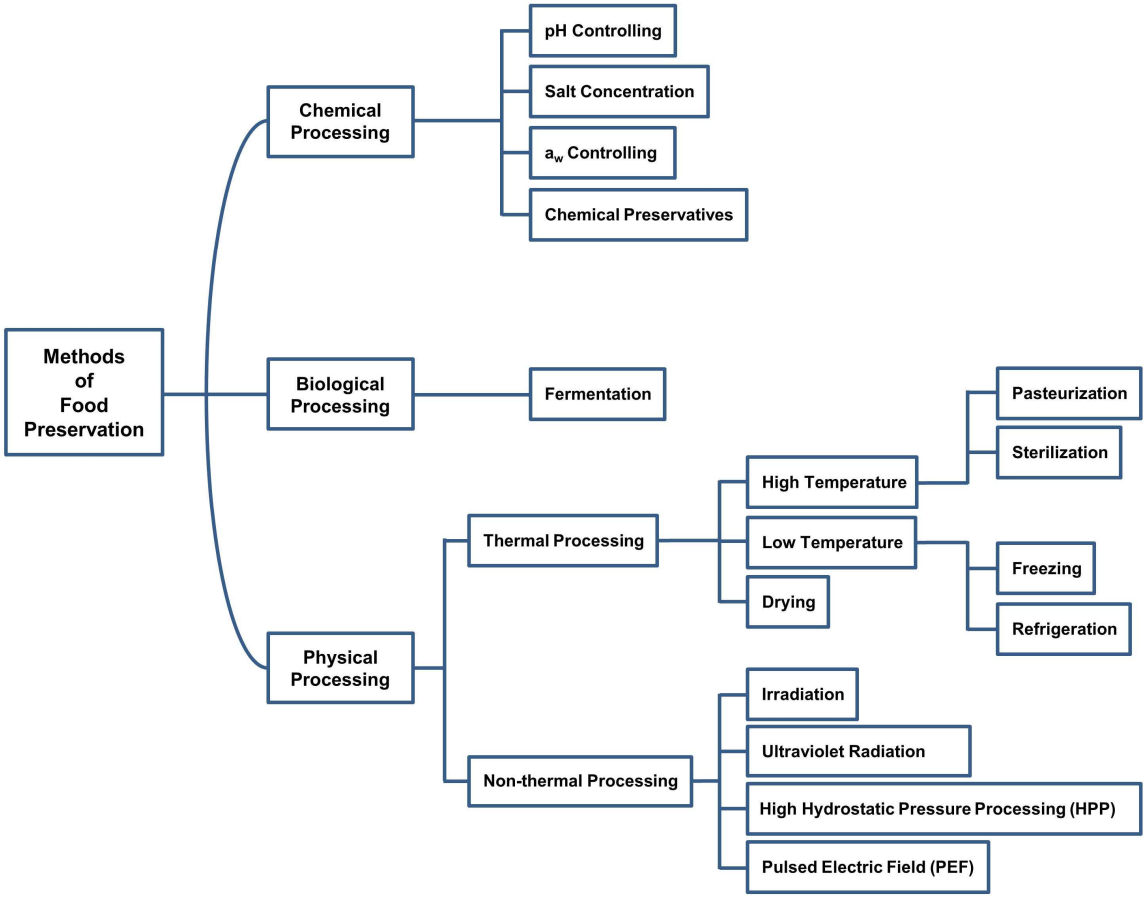

Figure 13. Classification of food preservation and processing methods.
Modified Figure from journal article [188].

The efficiency of the preservation methods for inactivation of pathogens is dependent on multiple factors, including the specific type of pathogen, the applied distinct processing parameters as well as the specific food matrix. Whereas many data are available on the inactivation of microorganisms, spores and enzymes, those are often lacking for viruses. For HEV in particular, very few data are available.

2.11 HEV inactivation – methods for investigation and current knowledge

In order to investigate inactivation of HEV due to physico-chemical treatments, the availability of laboratory assays for measurement of its infectivity is crucial. The widely used PCR methods, which represent the gold standard for detection and quantification of HEV RNA in clinical settings and research, are not suitable for HEV infectivity assessment. Therefore, many efforts have been done to develop efficient cell culture systems for HEV in the past, unfortunately with only limited success [191]. However, very recently improved cell culture systems became available, which use specific HEV strains and optimized cell lines in order to achieve better replication performances in cell culture [192], [193]. As a result, these improved cell culture systems are able to robustly determine HEV infectivity in solutions [62], [167], [192], [194]–[196]. One of these improved cell culture systems uses the HEV-3c strain 47832c, which efficiently replicates in persistently infected A549 cells [57]. For infection, A549 cells of subtype D3 are used due to their higher sensitivity to this HEV strain. A quantitative infectivity determination in a range of approximately 4 $\log_{10}$ is possible by virus titration in a 96-well plate format. Since HEV shows no cytopathic effect in cell culture, the evaluation is realized using immunofluorescence [57] (Fig. 14). Despite the advantages of the improved cell culture systems, all of them remain labor-intensive, time-consuming and thus expensive compared to many other viruses [197]. In addition, none of them can be used to determine HEV infectivity directly in food so far.

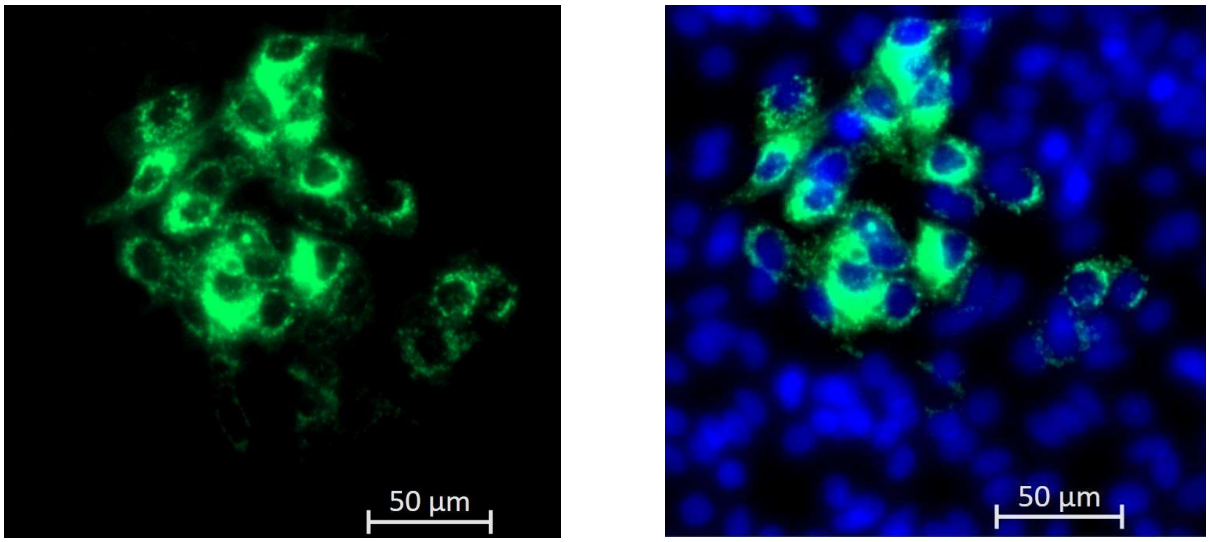

Figure 14. Inverse fluorescence microscopic images of HEV-3c strain 47832c-infected A549/D3 cells. The left image shows a so-called HEV focus, which consists of many adjacent infected cells. The virus antigen located in the cytosol was stained green, using primary rabbit anti-capsid and secondary fluorescein isothiocyanate (FITC)-labeled anti-rabbit goat antibodies and visualized with enhanced green fluorescent protein (EGFP) signal. The right image shows the same focus, but with additional blue 4′,6-diamidino-2-phenylindole (DAPI) staining of the cell nuclei. Bars: 50 µm.

Another possibility for infectivity testing of HEV are animal experiments. In this context the pig model using HEV-3 has proven to be particularly useful [198], [199]. However, only a limited number of animals can be used for those experiments, therefore limiting the tested conditions and sample replications. In addition, most animal models can only be successfully infected intravenously, which does not correspond to natural routes of transmission [200]. In the case of cynomolgus macaques, a non-human primate species, the minimal infectious dose between oral and intravenous inoculation diverges by at least a factor of 10,000, according to one study [201]. Furthermore, ethical concerns are becoming increasingly prominent. However, animal experiments are essential for questions that cannot be answered by other techniques. These include understanding *in vivo* characteristics of HEV infection, viral pathogenesis and efficiency testing of antivirals and vaccines [202], [203].

Very little is known about HEV stability and inactivation so far. In one study, inactivation in cell culture medium by short-term exposure to a range of heating

conditions and to long-term storage at three different temperatures was investigated using a cell culture method [204]. Two other studies investigated HEV inactivation in food by heat in pig models. The first study examined HEV inactivation in pig liver by exposure to moderate heat, stir-frying or boiling [198]. The other study examined HEV inactivation in pig liver pâté by heat exposure at three different temperatures and three different exposure times [199]. Among other physico-chemical parameters affecting HEV stability, only the effects of chlorine and ultraviolet (UV) light have been investigated so far, using cell culture methods in both studies [205], [206].

3 Importance of this thesis

Infections with HEV pose a major burden for humans worldwide. Whereas small and large outbreaks are consistently reported in developing regions caused by HEV-1 and HEV-2, the HEV genotypes 3 and 4 are of particular concern in developed regions, where they cause sporadic acute and chronic hepatitis E cases. HEV is considered as an endemic pathogen in many industrialized countries, and the numbers of notified hepatitis E cases are increasing in several European countries including Germany within the last two decades. The zoonotic HEV-3, which is mainly responsible for these HEV infections, circulates in indigenous reservoir animals such as pigs and wild boars. The main transmission route is assumed to be via consumption of meat and meat products from infected animals. However, the distinct risk for human infection by consumption of specific types of meat products cannot be assessed, as the HEV inactivation parameters during their production are not known. Also, the efficiency of specific methods applied during food production for the inactivation of HEV has not been assessed so far. In addition, the risk of contaminated surfaces for spreading of HEV could not be assessed, as the ability of its survival on the surfaces was not known. The missing data on HEV stability therefore hindered the development of more efficient measures and suggestions for effective prevention of HEV transmission by food.

At the beginning of this thesis, very little was known about the stability and inactivation of HEV in general. This included the HEV stability during heating and long-term storage in liquids, as well as against treatment with UV light and chlorine. These findings are particularly important for treatment of drinking water in developing countries and regions of crisis. However, the HEV stability against physical and chemical parameters normally applied in meat industry and present in private households as well as the environment were nearly completely unknown. This lack of data can be explained by the fact that sufficiently well-performing cell culture systems for HEV infectivity determination were only very limited available at the beginning of this thesis.

In order to address these questions, the specific objects of this thesis include:

- Optimization of the established cell culture system from Johne [204] with special regard to increase reproducibility and available virus titers, in order to provide a robust and suitable system for infectivity titration in inactivation studies.
- Further characterization of the obtained virus stock dispersions with regard to the ratio of quasi-enveloped and non-enveloped HEV particles in the mixtures to enable an assessment of comparability with natural contaminations.
- Assessment of HEV stability against a broad range of pH values and pH conditions typically used in raw sausage production.
- Assessment of HEV stability against high salt concentrations and salt conditions typically used for raw sausages.
- Assessment of HEV stability against high hydrostatic pressure processing, which is increasingly used for food preservation.
- Assessment of HEV stability against the drying process itself and during long-term storage of dried HEV at different surfaces, in order to estimate the risk of cross-contaminations in different settings including food production and environment.

The results of these studies should help to understand the effects of each parameter for HEV inactivation, which may thereafter be used for the development of mathematical models for the description of more complex scenarios. The results should also help to specify the risk of specific food products and to uncover potential additional transmission pathways of HEV. Furthermore, they should be used for the development of strategies to increase food safety by application of effective processing methods in order to reduce the presence of infectious virus in specific products.

4 Publications

4.1 List of publications and own contribution

Publication 1:

Stability of hepatitis E virus at different pH values

A. Wolff, T. Günther, T. Albert, K. Schilling-Loeffler, A.K. Gadicherla, R. Johne

International Journal of Food Microbiology (2020) 325:108625

https://doi.org/10.1016/j.ijfoodmicro.2020.108625

I took part in the conceptualization of the experiments. I was involved in producing the virus stock. I conducted both, the stability testing experiments including titration of residual infectivity and characterization of the virus stock. I evaluated the results and was involved in writing and editing of the manuscript.

Publication 2:

Effect of Sodium Chloride, Sodium Nitrite and Sodium Nitrate on the Infectivity of Hepatitis E Virus

Alexander Wolff · Taras Günther · Thiemo Albert · Reimar Johne

Food and Environmental Virology (2020) 12(4):350–354

https://doi.org/10.1007/s12560-020-09440-2

I took part in the conceptualization of the experiments. I was involved in producing the virus stock. I conducted the stability testing experiments including titration of residual infectivity. I evaluated the results and wrote the manuscript. I was involved in editing of the manuscript.

Publication 3:

Stability of hepatitis E virus at high hydrostatic pressure processing

R. Johne, A. Wolff, A.K. Gadicherla, M. Filter, O. Schlüter

International Journal of Food Microbiology (2021) 339:109013

https://doi.org/10.1016/j.ijfoodmicro.2020.109013

I was involved in producing the virus stock. I wrote parts of the manuscript and was involved in critical rewriting.

Publication 4:

Stability of Hepatitis E Virus After Drying on Different Surfaces

Alexander Wolff · Taras Günther · Reimar Johne

Food and Environmental Virology (2022) 14(2):138-148

https://doi.org/10.1007/s12560-022-09510-7

I took part in the conceptualization of the experiments. I was involved in producing the virus stock. I conducted the stability testing experiments including titration of residual infectivity. I evaluated the results and wrote the manuscript. I was involved in editing of the manuscript.

4.2 Publication 1: Stability of hepatitis E virus at different pH values

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However, it can be purchased by clicking on the DOI link on page 41.

Food and Environmental Virology (2020) 12:350–354
https://doi.org/10.1007/s12560-020-09440-2

BRIEF COMMUNICATION

Effect of Sodium Chloride, Sodium Nitrite and Sodium Nitrate on the Infectivity of Hepatitis E Virus

Alexander Wolff[1] · Taras Günther[1] · Thiemo Albert[2] · Reimar Johne[1]

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Abstract
Hepatitis E virus (HEV) infection can cause acute and chronic hepatitis in humans. The zoonotic HEV genotype 3, which is highly prevalent in Europe, is mainly transmitted by consumption of raw meat and raw meat products produced from infected pigs or wild boars. High salt concentrations represent an important measure to preserve meat products and to inactivate foodborne pathogens. Here, an HEV preparation in phosphate-buffered saline (PBS) was subjected to different salt concentrations and the remaining infectivity was measured in a cell culture assay. Treatments with up to 20% sodium chloride for 24 h at 23 °C, with and without addition of 0.015% sodium nitrite or 0.03% sodium nitrate, did not lead to virus inactivation as compared to PBS only. Conditions usually applied for short-term and long-term fermented raw sausages were simulated by incubation at 22 °C for up to 6 days and at 16 °C for up to 8 weeks, respectively. Only 2% sodium chloride with 0.015% sodium nitrite showed a weak (< 1 $\log_{10}$), but significant, infectivity reduction after 2 and 4 days as compared to PBS only. Addition of 2% sodium chloride and 0.03% sodium nitrate showed a slight, but not significant, decrease in infectivity after 2 and 8 weeks as compared to PBS only. In conclusion, HEV is highly stable at high salt concentrations and at salt conditions usually applied to preserve raw meat products.

Keywords Hepatitis E virus · Inactivation · Sodium chloride · Sodium nitrite · Sodium nitrate

Infection with the hepatitis E virus (HEV) can cause acute or chronic hepatitis in humans. Especially, pregnant women or patients with underlying liver diseases are at risk of severe courses of acute hepatitis. In addition, chronic infections with liver cirrhosis and extrahepatic manifestations such as neurologic disorders have been described in immunosuppressed patients (Narayanan et al. 2019). Steeply increasing numbers of notified hepatitis E cases have been recently reported in several European countries (Aspinall et al. 2017). HEV is a single-stranded RNA virus, which can form quasi-enveloped and non-enveloped particles. Both particle types are infectious in cell culture (Yin et al. 2016). Most human-pathogenic HEV strains can be classified into the genotypes 1 to 4 (Johne et al. 2014). Among those, genotypes 3 and 4 are zoonotic and prevalent in reservoir animals, such as wild boars and pigs (Pavio et al. 2017). The most important transmission route of these zoonotic genotypes is considered to be via consumption of undercooked meat or raw meat products from infected animals, which is supported by several case reports (Colson et al. 2010; Masuda et al. 2005; Matsuda et al. 2003). However, the distinct risk of infection by specific meat products is unknown so far. One reason for this is that the stability of HEV under different conditions used for food production and preservation is not fully understood. Salting of meat to reduce the water activity value is an important measure for preserving food, among other microbial hurdles (Leistner 2000). For salting of meat, nitrite or nitrate curing salt is commonly used to influence the color and the taste as well as to enhance the shelf life and safety of meat products. Nitrite exhibits an antimicrobial activity by inhibiting enzymes or disrupting electron transports in several microbes (Wirth 1980; Mueller-Herbst et al. 2014).

The aim of this study was to assess the stability of HEV against different salt concentrations at conditions usually applied during meat preservation. Due to the lack of reliable methods for measurement of HEV infectivity directly in meat products (Cook et al. 2017), the stability of HEV

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against different salts was analyzed here using pure virus suspensions. The experiments were performed at different temperatures and incubation times, simulating the conditions during short-term and long-term fermentation of raw sausages.

For stability experiments and residual infectivity titrations, an established cell culture system using the human HEV genotype 3c strain 47832c was used as described (Wolff et al. 2020). Briefly, a virus stock suspension was prepared by collecting the supernatant of persistently HEV-infected A549 cells after three freeze/thaw cycles, which results in a mixture of quasi-enveloped and non-enveloped virus particles (Wolff et al. 2020). These particles were then pelleted by ultracentrifugation and the pellet was resuspended in phosphate-buffered saline (PBS, PAN-Biotech GmbH, Germany). The resulting virus stock suspension had a concentration of 2.9×10^4 focus-forming units (ffu)/ml of infectious HEV particles. Salt stock solutions with varying concentrations of sodium chloride, sodium nitrite and sodium nitrate (Merck, Germany) were prepared in PBS. For the stability experiments, aliquots of 500 µl virus stock suspension were mixed with the salt stock solutions resulting in the desired salt concentrations at final volumes of up to 5 ml. All concentrations were calculated in percent by mass [m/m]. The mixtures were incubated at the indicated temperatures and time intervals. In order to exclude pH effects on HEV infectivity during the salt experiments, control salt mixtures without virus were measured with a micro pH electrode at days 0 and 7 as described (Wolff et al. 2020). At the desired time-points, each virus sample was diluted with PBS to a final volume of 20 ml to stop the incubation. Thereafter, the samples were ultrafiltrated using Vivaspin 20 ultrafiltration tubes (50 kDa MWCO, PES membrane, Sartorius, Germany) to a final volume of 500 µl and stored at 4 °C until infectivity titration on the same day, which was performed exactly as described (Johne et al. 2016). Briefly, tenfold dilutions of the samples were used for infection of A549/D3 cells in a 96-well plate format. After 2 weeks, infected cells were stained, using an HEV capsid protein-specific rabbit hyperimmune serum followed by a FITC-conjugated antirabbit IgG antibody, and visualized using an inverse fluorescence microscope. The number of fluorescence foci was counted manually and infectivity values were calculated in ffu/ml. Each experimental condition was analyzed in 2 independent biological replications with 4 technical replications each. The infectivity values were $\log_{10}$-transformed and thereafter statistically analyzed. Statistical tests as for normal distribution (Shapiro–Wilk test and q–q plots), general difference between all samples (Kruskal–Wallis test) and specific differences between individual samples (pairwise Wilcoxon test for unpaired samples) were performed in R 3.5 (R Core Team 2019). In all statistical tests the significance level was set to $\alpha = 5\%$.

In the first experiment, HEV was subjected to extreme salt concentrations for 24 h at 23 °C (Fig. 1). In detail, the HEV stock suspension was treated with sodium chloride at concentrations of 2%, 10% or 20%. As a control reaction without adding of salt, only the HEV stock suspension (HEV in PBS containing 0.8% sodium chloride) was used. Some of the samples were treated additionally with 0.015% sodium

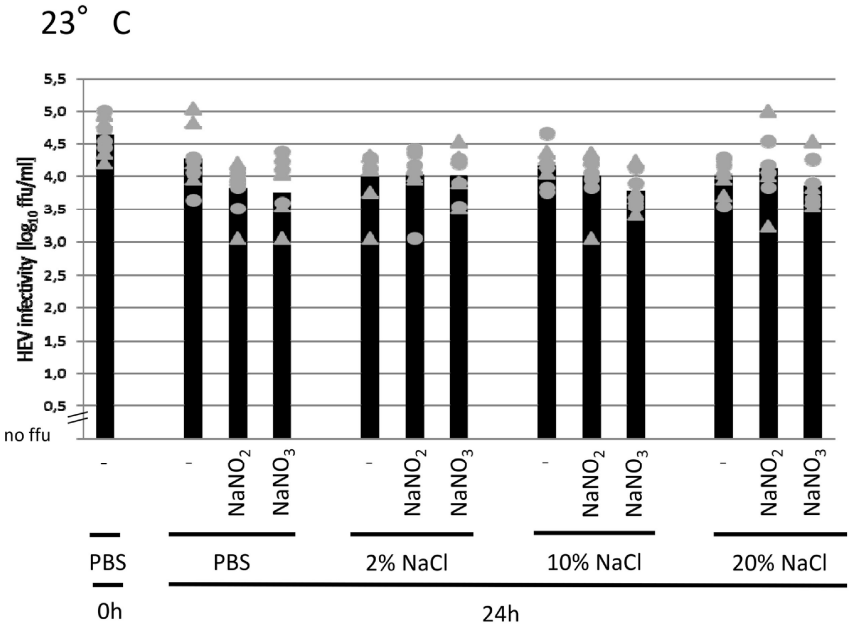


Fig. 1 Infectivity of HEV after treatment with high salt concentrations. The samples were incubated with the indicated salt concentrations in phosphate-buffered saline (PBS) for 24 h at 23 °C. The infectious virus titers were titrated on A549/D3 cell cultures. The infectivity values, generated from two independent treatments (first treatment gray circles and second treatment gray triangles) with 4 replications each, and the arithmetic means (black columns) are indicated in $\log_{10}$ focus-forming units/ml. No significant differences were detected between all samples after 24 h. NaCl: sodium chloride at the indicated concentrations; $NaNO_2$: sodium nitrite (0.015%); $NaNO_3$: sodium nitrate (0.03%)

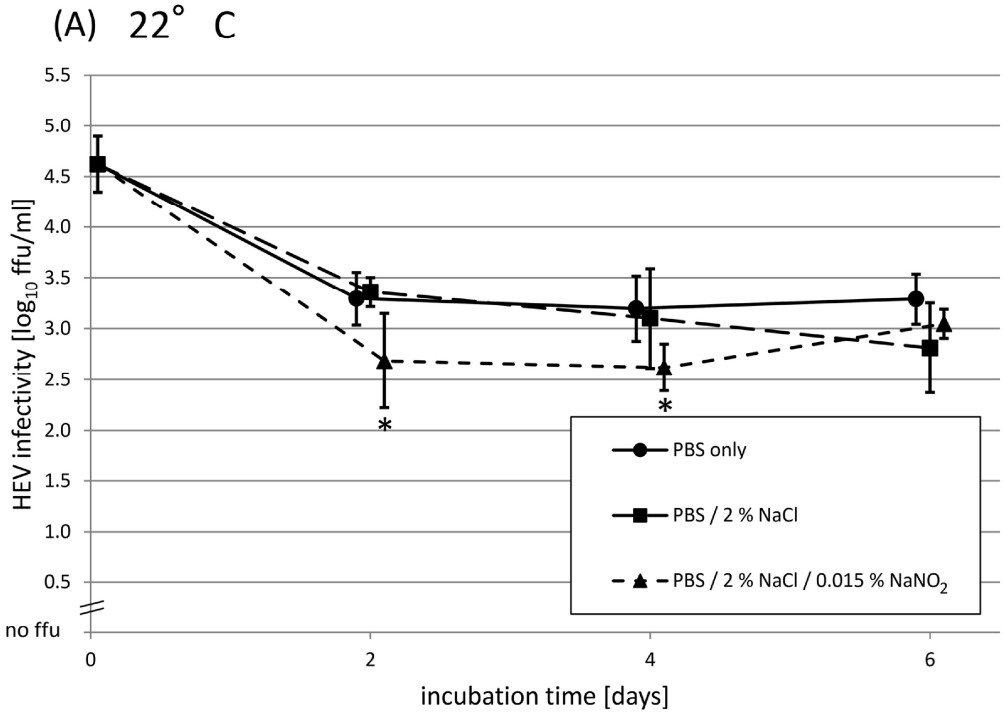
nitrite or 0.03% sodium nitrate. After the incubation time, the arithmetic mean of all incubated samples was 0.6 $\log_{10}$ ffu/ml lower than the arithmetic mean of the non-incubated sample. However, no significant differences were observed between all incubated samples irrespectively of their salt concentrations. The results of the experiment indicate that HEV exhibits a very high resistance to various salt concentrations and conditions. Even at a concentration of 20% sodium chloride, which is much higher than those commonly used in food, and concentrations of sodium nitrite and sodium nitrate representing the upper limits allowed due to European legislation on food additives (Regulation (EC) No 1333/2008), the virus turned out to be stable during the short time incubation analyzed here.

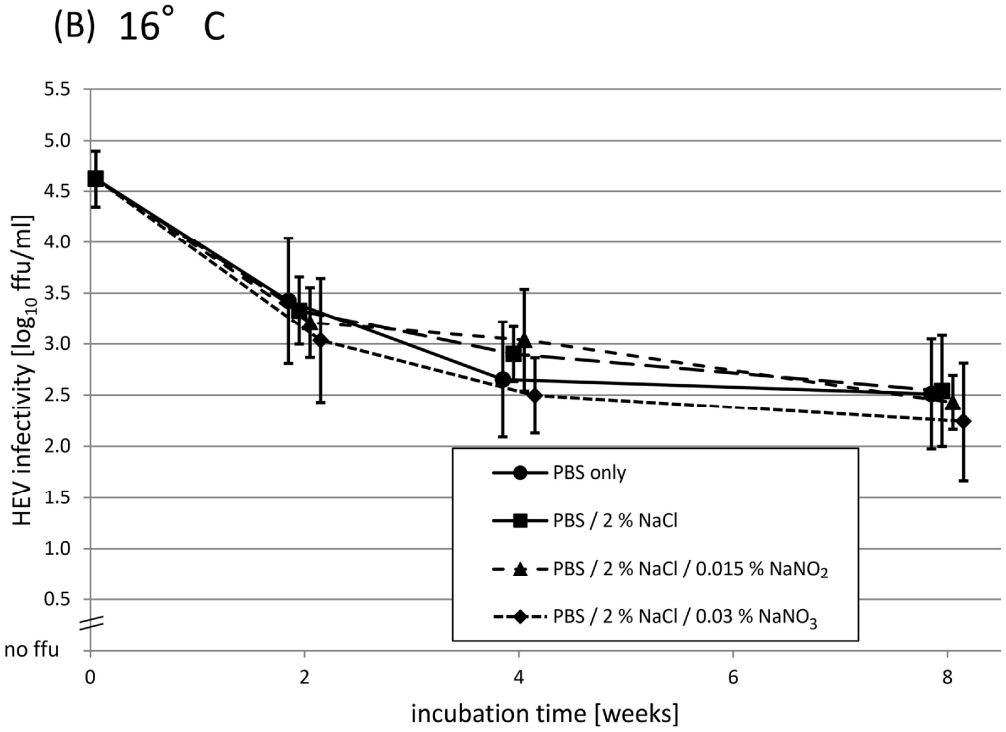
In the second experiment, HEV was subjected to salt conditions which normally occur during short-term fermentation of raw sausages (Fig. 2a). Incubation was done for up to 6 days at 22 °C which is typical for production of this sausage type (Keim and Franke 2007). In detail, the HEV stock suspension was treated with 2% sodium chloride or 2% sodium chloride with 0.015% sodium nitrite and compared to the HEV stock suspension in PBS only. The mean infectivity decreased during six days of incubation by 1.6 $\log_{10}$ ffu/ml as compared to the initial value, with a rapid loss in the first two days followed by a plateau phase. Overall, only minor differences between all conditions could be observed. Only the condition with sodium chloride and sodium nitrite at days 2 and 4 showed significantly lower mean titers as compared to the others, although the observed differences were low (< 1 $\log_{10}$ ffu/ml). In detail, significant differences were found for 2% sodium chloride with 0.015% sodium nitrite vs. PBS only for after 2 ($p = 0.037$) and 4 ($p = 0.028$) days of incubation, and for 2% sodium chloride with 0.015% sodium nitrite vs. 2% sodium chloride for day 2 ($p = 0.028$). In the pH control samples, a decrease of pH values (from a maximum of pH 7.5 to a minimum of pH 6.4) due to an increase of sodium chloride was found, whereas the effect of sodium nitrite and sodium nitrate in the mixtures was negligible. After 7 days of storage at 22 °C, all pH values decreased slightly, with an arithmetic mean pH decrease of 0.03. It has been shown recently, that only pH values < 5 had significant effects on HEV infectivity after storage for 7 days at room temperature (Wolff et al. 2020); therefore, the pH effect can be considered minimal here. In summary, the experiment showed that salt conditions commonly occurring during short-term fermentation of raw sausages have no or only very weak effects on HEV inactivation.

In the third experiment, HEV was subjected to salt conditions which normally occur during long-term fermentation of raw sausages (Fig. 2b). Incubation was done for up to 8 weeks at 16 °C which is typical for production of this sausage type (Keim and Franke 2007). In detail, the HEV stock suspension was treated with 2% sodium chloride, 2% sodium chloride with 0.015% sodium nitrite or 2% sodium chloride with 0.03% sodium nitrate in comparison to the HEV stock suspension in PBS only. The mean infectivity decreased about 1.8 $\log_{10}$ ffu/ml during the whole incubation time period of 8 weeks compared to the initial value, with a rapid decrease until week 2, followed by a weaker decrease. No obvious differences between all conditions could be observed. However, a general, but not significant, trend to lower mean values of infectivity for the treatment with sodium chloride and sodium nitrate was found. Generally, it has to be concluded from this experiment that even with longer incubation times, the salt conditions have no obvious effect on HEV inactivation.

The stability of other viruses against salt conditions usually occurring during food preservation has been only scarcely analyzed so far. Enteric cytopathic human orphan (ECHO) virus, a surrogate for human enteroviruses, also shows a very high resistance against extreme concentrations of sodium chloride. After 7 days of exposure to a 20% sodium chloride solution at 4 °C or 20 °C, no inactivating effect was found (Straube et al. 2011). In contrast, the infectivity reduction of feline calicivirus (FCV), a surrogate for human noroviruses, correlated with higher sodium chloride concentrations, longer incubation times and higher temperatures. After 3 h of incubation with sodium chloride concentrations up to 20% at 4 °C or 20 °C, no significant loss of virus infectivity could be detected compared to the PBS control. However, virus titers decreased significantly after 7 days of incubation at higher temperature (20 °C), with stronger reduction by 6%, 12% and 20% sodium chloride as compared to 2% and the PBS control (Straube et al. 2011). Adding of 0.01%, 0.015% or 0.02% sodium nitrite to a 2% sodium chloride solution showed no effect on infectivity reduction of FCV or ECHO virus (Straube et al. 2011). It can be concluded from this comparison, that HEV behaves similar to ECHO virus with regard to salt stability, but FCV should therefore not be considered as surrogate for HEV in stability experiments involving salts.

In conclusion, our study showed that HEV is highly stable at different salt concentrations. The results indicate that HEV will not be efficiently inactivated at salt conditions occurring during short-term or long-term fermentation of raw sausages. Another parameter commonly used for preservation of raw sausages is lowering of the pH value. However, it has been shown recently that HEV is also highly stable against a large range of different pH values, including those usually occurring during fermentation processes (Wolff et al. 2020). Taken together, it has to be considered that residual infectious virus will still be present in fermented meat products, if sufficiently high HEV-contaminated meat was used as starting material. One limitation of the study is that the analysis of the HEV salt stability was done in a liquid solution, which may not completely reflect the situation in meat


(A) 22° C
HEV infectivity [log10 ffu/ml]
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
no ffu
0
2
4
6
incubation time [days]
*
*
PBS only
PBS / 2 % NaCl
PBS / 2 % NaCl / 0.015 % NaNO2


(B) 16° C
HEV infectivity [log10 ffu/ml]
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
no ffu
0
2
4
6
8
incubation time [weeks]
PBS only
PBS / 2 % NaCl
PBS / 2 % NaCl / 0.015 % NaNO2
PBS / 2 % NaCl / 0.03 % NaNO3

◂**Fig. 2** Time-courses of HEV infectivity after incubation at different salt conditions. **a** Treatment at 22 °C for up to 6 days; **b** treatment at 16 °C for up to 8 weeks. The samples were incubated with phosphate-buffered saline (PBS) only (black circles), PBS containing 2% sodium chloride (NaCl) (black squares), PBS containing 2% sodium chloride and 0.015% sodium nitrite ($NaNO_2$) (black triangles) or PBS containing 2% sodium chloride and 0.03% sodium nitrate ($NaNO_3$) (black diamonds). The infectious virus titers were titrated on A549/D3 cell cultures. The infectivity values shown as arithmetic means from two independent treatments with 4 replications each are indicated in $\log_{10}$ focus-forming units/ml, as well as their standard deviations. *Significant differences ($p < 0.05$)

products. Future investigations should therefore focus on direct measurement of HEV infectivity in the meat matrix, in order to validate the findings of this study.

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4.4 Publication 3: Stability of hepatitis E virus at high hydrostatic pressure processing

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Stability of hepatitis E virus at high hydrostatic pressure processing

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ABSTRACT

Hepatitis E virus (HEV) is the causative agent of acute and chronic hepatitis in humans. The zoonotic HEV genotype 3 is the main genotype in Europe. The foodborne transmission via consumption of meat and meat products prepared from infected pigs or wild boars is considered the major transmission route of this genotype. High hydrostatic pressure processing (HPP) is a technique, which can be used for inactivation of pathogens in food. Here, preparations of a cell culture-adapted HEV genotype 3 strain in phosphate-buffered saline (PBS) were subjected to HPP and the remaining infectivity was titrated in cell culture by counting fluorescent foci of replicating virus. A gradual decrease in infectivity was found by application of 100 to 600 MPa for 2 min. At 20 °C, infectivity reduction of 0.5 $\log_{10}$ at 200 MPa and 1 $\log_{10}$ at 400 MPa were observed. Slightly higher infectivity reduction of 1 $\log_{10}$ at 200 MPa and 2 $\log_{10}$ at 400 MPa were found by application of the pressure at 4 °C. At both temperatures, the virus was nearly completely inactivated (>3.5 $\log_{10}$ infectivity decrease) at 600 MPa; however, low amounts of remaining infectious virus were observed in one of three replicates in both cases. Transmission electron microscopy showed disassembled and distorted particles in the preparations treated with 600 MPa. Time-course experiments at 400 MPa showed a continuous decline of infectivity from 30 s to 10 min, leading to a 2 $\log_{10}$ infectivity decrease at 20 °C and to a 2.5 $\log_{10}$ infectivity decrease at 4 °C for a 10 min pressure application each. Predictive models for inactivation of HEV by HPP were generated on the basis of the generated data. The results show that HPP treatment can reduce HEV infectivity, which is mainly dependent on pressure height and duration of the HPP treatment. Compared to other viruses, HEV appears to be relatively stable against HPP and high pressure/long time combinations have to be applied for significant reduction of infectivity.

1. Introduction

The hepatitis E virus (HEV) is the etiologic agent of an acute hepatitis in humans. Large outbreaks of hepatitis E occurred in developing countries due to contaminated drinking water, whereas sporadic cases are predominant in industrialized countries (Goel and Aggarwal, 2020). In Europe, the number of notified hepatitis E cases increased during the last years (Aspinall et al., 2017). In addition to acute hepatitis, chronic disease courses, which may develop to life-threatening liver cirrhosis, have been increasingly described in immunosuppressed transplant patients (Narayanan et al., 2019).

HEV is a small virus with a diameter of 40–50 nm, which has a single-stranded RNA genome. The virus particles exist in two forms: non-enveloped particles are excreted by feces whereas quasi-enveloped particles have been identified in serum and cell culture supernatant (Yin et al., 2016). Both particle types are infectious in cell culture. Within the family *Hepeviridae*, four major human-pathogenic genotypes have been identified. Whereas genotypes 1 and 2 exclusively infect humans, genotypes 3 and 4 are zoonotic and have a large animal reservoir in pigs and wild boars (Pavio et al., 2017). Foodborne transmission via consumption of meat and meat products prepared from infected animals is considered the major transmission route for genotypes 3 and 4. In Europe, the genotype 3 subtypes 3c and 3f are circulating predominantly in humans and animals (Abravanel et al., 2020).

The efficiency of inactivation methods for HEV during meat processing is largely unknown. The lack of efficient and easy-to-use methods for infectivity determination of HEV prevented larger inactivation studies for HEV in the past. Although significant progress has been made in cell culture propagation of HEV during the last years, a robust and reproducible system for HEV inactivation studies directly in meat products is still missing (Cook et al., 2017). Recently, the stability of HEV in cell culture medium and PBS by application of short heating,

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long-term storage, different pH values and salt concentrations has been analyzed, which may be used to predict its behavior during production of different meat products (Johne et al., 2016; Wolff et al., 2020a; Wolff et al., 2020b). The HEV genotype 3c strain 47832c (Johne et al., 2014) has been used in these studies in a cell culture system, in which virus-infected cells are visualized by immunofluorescence as focus-forming units (ffu) that directly relate to the amount of infectious HEV particles in a sample.

The suitability of high hydrostatic pressure processing (HPP) for inactivating microorganisms and denaturing proteins was already demonstrated more than a hundred years ago (Bridgman, 1914; Hite, 1899). For several decades now, the industrial application of high hydrostatic pressure has been established as a gentle alternative to thermal treatment, whereby various food products are treated, such as juice and beverages, vegetable products, meat products and seafood (Huang et al., 2017). The pressure effect is vectorially undirected and therefore uniform throughout the system, in contrast to the large temperature gradients that occur during conventional thermal treatment of food. The inactivating effect of HPP is mainly attributed to protein denaturation, pH change, dissociation of ribosomes (Molina-Gutierrez et al., 2002; Mota et al., 2013) as well as phase transitions and changes in fluidity of cell membranes and cell membrane permeabilisation (Winter and Jeworrek, 2009). However, matrix effects must be considered as well.

HPP treatment has also been shown to be efficient for inactivation of several viruses like human norovirus (Leon et al., 2011), rotavirus (Araud et al., 2015) and hepatitis A virus (Calci et al., 2005). A first study on inactivation of HEV by HPP treatment has been published recently, indicating an unusually high stability of this virus (Nasheri et al., 2020). In this study, the HEV strain 47832c was used as well; however, an indirect method quantifying HEV-RNA in the supernatant of inoculated cell culture was applied for infectivity determination.

To further elaborate the stability of HEV at HPP treatment, HEV-containing samples were investigated here at different pressure/time combinations. To enable compatibility with formerly published inactivation data on HEV, the treatments were performed in PBS and residual infectivity was assessed by measuring focus-forming units in cell culture. Different pressure conditions were compared to each other by applying the same holding time at two different temperatures. In addition, the inactivation kinetics at a fixed pressure over a range of longer holding times was analyzed. The data were used for the generation of a predictive model, which can be used to predict HEV stability for any pressure/holding time/temperature combination within the analyzed experimental conditions.

2. Materials and methods

2.1. Virus and cells

The HEV genotype 3c strain 47832c (GenBank acc. no. KC618403), originally derived from a chronically infected patient (Johne et al., 2014), was used for all assays, described in this paper. For virus production, an A549 cell line persistently infected with this strain (Johne et al., 2014; Johne et al., 2016) was used. The subclonal cell line A549/D3, originally derived from A549 cells (Johne et al., 2016; Schemmerer et al., 2016), which shows a higher susceptibility to this virus strain compared to normal A549 cells, was used for infectivity titration.

2.2. Virus production

The protocol to obtain a high viral load stock dispersion was used here as described in Wolff et al. (2020a), with slight modifications. Briefly, flasks with the persistently infected and completely confluent cells were frozen at −20 °C and thawed 3 times, before their supernatants were harvested. In order to concentrate the virus, the pooled supernatants were subjected to ultracentrifugation as described (Wolff et al., 2020a). The resulting virus pellets were resuspended in one-fiftieth of their starting volume in PBS. All virus suspensions generated in this way were pooled, aliquoted and stored at −20 °C until further use. After thawing, the virus stock suspension had a concentration of infectious HEV particles of 4.1×10^4 ffu/ml.

2.3. HPP treatment

The high pressure experiments were conducted in a single vessel unit (U111, UNIPRESS, Warszawa, Poland) using a hand piston pump for generation of pressure. The unit can operate at high hydrostatic pressure up to 0.9 GPa in a temperature range from −20 °C to 120 °C. The high pressure medium was 1,2-propanediol (≥99.5%, ROTH, Germany) mixed with distilled water (50% v/v). An externally piped small cylindrical pressure vessel made of high strength beryllium copper alloy (inner volume: 6 ml, inner diameter: 13 mm, inner height: 45 mm) was used. To determine the temperature profiles, the tip of a thermocouple was fixed inside the treatment unit directly above the sample tube and temperature changes during the pressure build-up phase, the pressure holding time and the pressure release were recorded (measurement frequency of 0.5 Hz, Supplementary Fig. 1). Thermocouples and pressure gauge were connected to the measurement system (ALMEMO 2590, AHLBORN, Germany) and data were recorded every 2 s. To control the temperature, the vessel was directly inserted into a thermal water bath (CC 410, Huber, Germany).

A total of 580 µl of the virus suspension was placed into 0.5 ml cryotubes (Nunc, Thermo Scientific, Germany) under sterile conditions and the closed sample tube was then transferred into the vessel at operation temperature. In a first series of experiments, pressures of 100 to 600 MPa were applied for a fixed time of 2 min at 20 °C or 4 °C. In a second series of experiments, treatments were done at a fixed pressure of 400 MPa at holding times between 0.5 and 10 min at 20 °C or 4 °C. Each pressure/time combination was analyzed by three replicates, which were separately subjected to HPP treatment. The pressure was manually increased (at average rates between 1 MPa/s and 35 MPa/s) to the required pressure level and then the holding time started. Pressure increase to 600 MPa (20 °C starting temperature) resulted in a maximum temperature of 24.1 °C due to quasi-adiabatic heating effects during the pressure increase. The pressure holding time was stopped by manual pressure release (ca. 10 MPa/s). During this phase, the temperature decreased temporarily, with a maximum decrease to −2.1 °C in the case of 600 MPa pressure application at 4 °C starting temperature. The average temperature (±SD) over all pressure conditions for 4 °C replicates was 3.9 ± 0.6 °C and for 20 °C replicates 19.2 ± 0.7 °C. The average pressure over all temperature conditions was 102.7 ± 1.2 MPa, 202.6 ± 0.8 MPa, 301.2 ± 2.2 MPa, 403.4 ± 1.3 MPa, 505.5 ± 1.1 MPa and 603.1 ± 2.6 MPa in the respective experiments. The vessel was unloaded and the treated sample tube was placed on ice until infectivity titration. Control samples were not treated with pressure and directly stored on ice.

2.4. Infectivity titration

HEV infectivity of the samples was determined by the established titration method, as described in detail in Johne et al. (2016). Briefly, tenfold-dilution series of the HEV samples were used to infect A459/D3 cells in a 96 well plate format. After 1 h of incubation, the supernatants of the cells were replaced by cell culture medium. Thereafter, the 96 well plates were incubated for 1 week in a humidified incubator, followed by an exchange of the medium and a second incubation period for 1 week. Afterwards, infected cells were stained by immunofluorescence, using a rabbit anti HEV capsid protein antiserum and FITC-labelled anti rabbit IgG secondary antibodies. Fluorescent foci were counted manually with an inverse fluorescence microscope. Infectivity values in ffu/ml were calculated on the basis of the numbers of counted fluorescent foci from the well of the highest dilution showing fluorescence, multiplied with the respective dilution factor using the MS Excel software.

2.5. Statistical analysis of data

Statistical analyses of the full set of experimental results were performed using the R environment for statistical computing version 3.6.3 (R Core Team, 2019) with the package "multcomp" v1.4–13 (http://multcomp.R-forge.R-project.org). The effects of experimental factors "holding time", "pressure" and "temperature" were analyzed through a multi-factorial ANCOVA using "pressure" and "temperature" as independent factors without interactions and "holding time" as co-variable. To identify significant differences exerted by individual "pressure" and "temperature" factor levels on the residual HEV activity the comparisons of factor level means were performed with glht() function applying the Bonferroni multiplicity adjustment (Bonferroni, 1936). The family wise error rate was controlled at a significance level of alpha = 5%. To check for compliance to the ANCOVA assumptions diagnostic plots on heteroscedasticity, normality, and influential observations were generated and inspected. All generated R code is available within the KNIME data analysis workflow (see 2.6).

2.6. Development of predictive models

For generation of a predictive HEV inactivation model we used the free, open source software KNIME Analytics Platform version 4.1.3 (KNIME AG, Zurich, Switzerland, www.knime.com) with the KNIME extension "PMM Nodes" (version 1.2.2.202008191506, German Federal Institute for Risk Assessment (BfR), https://foodrisklabs.bfr.bund.de/pmm-lab/). The model generation process followed the so-called "one-step fitting" approach (see Jewell, 2012). Visual inspection of available HEV inactivation data over various holding times led to the decision to apply a bi-phasic primary model equation (as described by Juneja et al., 2010) for describing the holding time-dependent reduction of HEV infectivity at constant environmental conditions. Due to the scarcity of experimental data, the parameter "intersection time" of the biphasic model equation was fixed to 2 min. The influence of environmental factors "temperature" and "pressure" was modelled via log-linear equations for the two primary model inactivation rates "D1" and "D2". For this, we adapted equation #5 from Farakos and Zwietering (2011); here, parameters "Pref" were fixed to 600 MPa and "Tref" fixed to 20 °C. For fitting the combined formula through the measured data points in a one-step approach, the KNIME extension "PMM-Lab" was used. PMM-Lab performed a maximum-likelihood-based search through the high-dimensional parameter space as described by Lorimer and Kiermeier (2007) starting from the 100 best sets of parameter start values. To avoid overfitting the initial model equation was simplified by an iterative backwards parameter elimination procedure, where non-significant model parameters were deleted one at a time. The process of model parameter elimination was continued until all model parameter estimates were significantly different from "0". This process resulted in the following final model equation:

$$Value = Y_0 - \left(\frac{Time}{10^{\,log10D_{PrefTref} + \left(\frac{600-pressure}{z_P}\right) - \left(\frac{20-T}{z_T}\right)}} \right) \frac{-(Time > 2)*(Time - 2)}{const} \frac{+(Time > 2)*(Time - 2)}{\left(10^{\,log10D_{PrefTref} + \left(\frac{600-pressure}{z_P}\right) - \left(\frac{20-T}{z_T}\right)} \right)}$$

Here "Value" represent the predicted residual infectivity in $\log_{10}$ (ffu/ml) after treating the sample for a distinct holding time "Time" (in seconds) at constant "pressure" conditions (in MPa). "Y_0" represents the initial infectivity at "Time" = 0 min; "const", "$\log10D_{PrefTref}$", "z_P" and "z_T" are model parameters fitted during the model generation process, where "const" represent the rate of HEV inactivation in the second inactivation phase (i.e. after 2 min holding time); "$\log10D_{PrefTref}$" is the $\log_{10}$-transformed time needed for a 1 log infectivity reduction (D-value) for pressure inactivation at 600 MPa and 20 °C, "z_P" and "z_T" are the change in pressure / temperature needed to reduce the D-value by 90% (1 $\log_{10}$ reduction of "log10D"). The complete model generation process is documented in a KNIME workflow that is provided upon request. The final predictive model for HEV inactivation is further exported into the software-independent model exchange format PMF-ML (http://sourceforge.net/projects/microbialmodelingexchange/), i.e. as a PMFX file that holds all model parameter estimates, the raw experimental data and all relevant metadata, including a description of the model's range of applicability. The generated file can be accessed via the following model repository: http://data.d4science.org/ctlg/RAKIP_portal/hepatitis_e_virus_inactivation_model_for_hpp_treatment.

2.7. Electron microscopy

10 µl of supernatant from either pressure treated or untreated viral suspension was adsorbed on to 400 mesh carbon-formvar coated copper grids (Plano GmbH, Germany) for 5 min followed by fixation with glutaraldehyde for a further 1 min. Excess liquid was removed by passive capillary action using a tissue paper. The grids were then contrasted with 2% uranyl acetate for 1 min and excess liquid removed as before. The grids were allowed to dry and examined in a Jeol 1400 Plus TEM (Jeol, Japan), operated at 120 kV. Six different areas of the grid were examined to check the homogeneity of sample. Imaging was performed with Olympus Veleta G2 camera (EMSIS, Germany). Particle diameter was measured using ITEM software provided by Olympus.

3. Results

3.1. HEV treatment at 100 to 600 MPa for 2 min

HEV preparations in PBS were treated at two different temperatures for 2 min at atmospheric pressure and pressures between 100 MPa and 600 MPa. At 4 °C as well as at 20 °C, gradually decreasing amounts of remaining infectious HEV were determined by increased pressure conditions (Fig. 1). A slightly higher degree of inactivation was evident at 4 °C as compared to 20 °C. In detail, mean infectivity decreases compared to control treatments under atmospheric pressure of about 0.5 $\log_{10}$ ffu/ml at 200 MPa, 1 $\log_{10}$ ffu/ml at 400 MPa and >3.5 $\log_{10}$ ffu/ml at 600 MPa were found at 20 °C. At 4 °C, mean decreases of about 1 $\log_{10}$ ffu/ml at 200 MPa, 2 $\log_{10}$ ffu/ml at 400 MPa and >3.5 $\log_{10}$ ffu/ml at 600 MPa were determined. At 600 MPa, only a single focus-forming unit was found in one of the three replicates at both temperatures.

3.2. HEV treatment at 400 MPa for different holding times

The dependency of HEV inactivation on pressure holding time was analyzed at 400 MPa, which represents a pressure widely used for HPP treatment of food. Again, the experiments were done at the two temperatures 4 °C and 20 °C. At pressure holding times between 30 s and 10 min, the residual amount of infectious virus decreased continuously, with a higher inactivation rate at the beginning and a lower additional inactivating effect at longer incubations (Fig. 2). Again, HEV was more efficiently inactivated at 4 °C as compared to 20 °C. In detail, mean infectivity decreases of about 0.5 $\log_{10}$ ffu/ml after 1 min, 1.5 $\log_{10}$ ffu/ml after 5 min and 2 $\log_{10}$ ffu/ml after 10 min were found at 20 °C. At 4 °C, mean decreases of about 1 $\log_{10}$ ffu/ml after 1 min and about 2.5 $\log_{10}$ ffu/ml after 5 or 10 min were determined. Residual infectious virus was detected in all replicates at the longest holding time of 10 min at both temperatures.

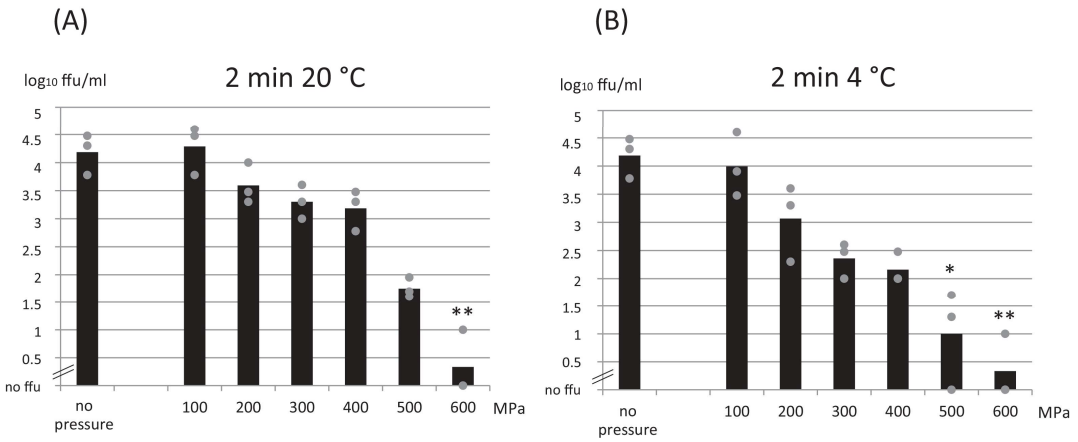

Fig. 1. Inactivation of HEV by treatment at atmospheric pressure and selected HPP conditions from 100 MPa to 600 MPa. HEV preparations in PBS were subjected to treatment at the indicated pressures for 2 min, at (A) 20 °C or (B) 4 °C. The residual amount of infectious HEV was determined by a cell culture method counting focus-forming units (ffu) per ml. Three biological replicates were treated independently, each was thereafter analyzed once in cell culture. The measured residual infectious HEV for each replicate (gray circles) and the arithmetic mean for each treatment condition (black columns) are shown. Significant differences (*$p < 0.05$, **$p < 0.01$) between HPP-treated and non-treated (atmospheric pressure) samples are indicated.

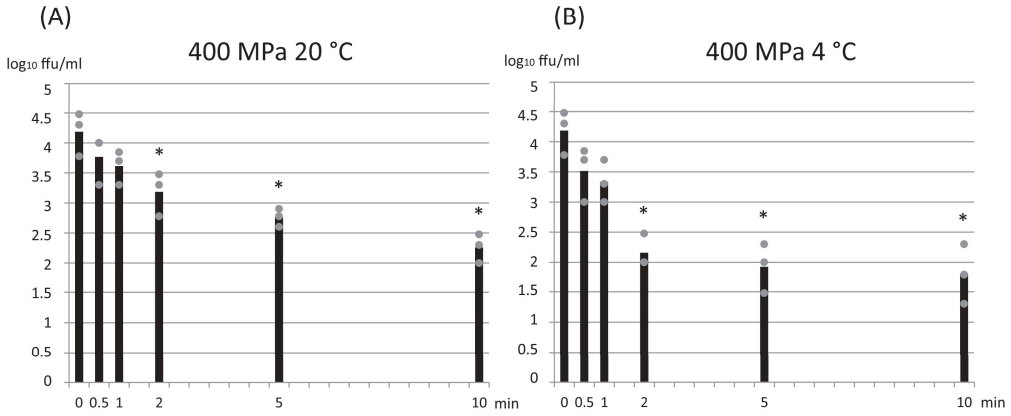

Fig. 2. Time-course of HEV inactivation during HPP treatment at 400 MPa. HEV preparations in PBS were subjected to HPP treatment for the indicated duration at (A) 20 °C or (B) 4 °C. The residual amount of infectious HEV was determined by a cell culture method counting focus-forming units (ffu) per ml. Three biological replicates were treated independently, each was thereafter analyzed once in cell culture. The measured residual infectious HEV for each replicate (gray circles) and the arithmetic mean for each treatment duration (black columns) are shown. Significant differences (*$p < 0.01$) between HPP-treated and non-treated (duration = 0 min) samples are indicated. For this, a post-hoc multiple comparison of means with *p*-values adjusted by the Bonferroni method was applied, where different holding times were considered as independent factor levels within the general linear model.

3.3. Statistical analysis and predictive modelling

The statistical analysis confirmed that the environmental factor "pressure" and the "holding time" had the strongest effect on HEV inactivation (both factors with p-values $<10^{-8}$) (Supplementary Fig. 2). The influence of temperature was also statistically significant (p-value = 0.04), but by far not as strong as the two other. There was no significant interaction effect between "temperature" and "pressure". From the post-hoc pairwise comparison of different pressure levels it can be concluded that 600 MPa resulted in significantly higher HEV inactivation compared to all other pressure levels below 500 MPa. The 500 MPa treatment still showed a significant higher inactivation compared to the 100 MPa and the control treatment (atmospheric pressure).

Based on the data, a new predictive model for HEV inactivation was generated. This HEV inactivation model (Fig. 3) showed a satisfactory accuracy with an overall root mean squared error (RMSE) of 0.42 $\log_{10}$ ffu/ml ($R^2 = 0.88$) for the measured experimental data. The fitted parameter estimates of the model are: $Y_0 = 4.23 + -0.06$; const = 16.4 ± 6.1; $\log 10D_{PrefTref} = -0.235 \pm 0.024$; $z_P = 549.4 \pm 40$ and $z_T = 141.3 \pm 31.4$. It is noteworthy that the final model also has "pressure", "temperature" and "holding time" as input parameters. This is in line with the results from the statistical analysis.

3.4. Electron microscopy of treated and untreated samples

To get more inside into the mechanism of HEV inactivation through HPP treatment, an HEV preparation without pressure treatment and one after treatment at 600 MPa for 2 min at 20 °C was analyzed by TEM. As shown in Fig. 4A and C, small particles with diameters of 40–50 nm resembling HEV particles were identified in the untreated preparation. In the pressure-treated preparation, those particles were not identified (Fig. 4B and D). In contrast, aggregates of smaller structures most

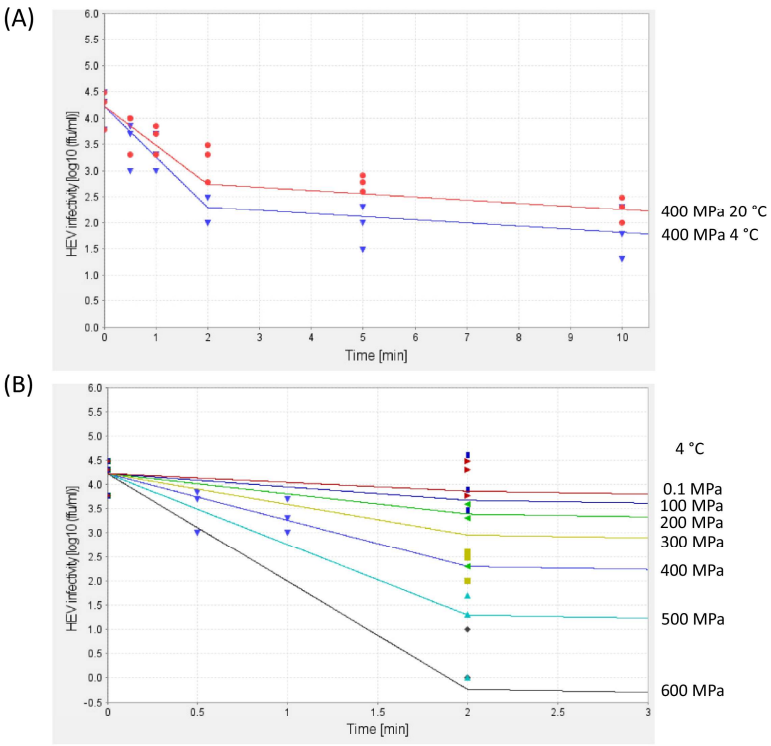

Fig. 3. Visualization of the prediction results for the HEV inactivation model illustrating the bi-phasic nature of the primary model equation with the parameter "intersections time" = 2 min (A) for the two experiments (20 °C and 4 °C) at 400 MPa with different holding times and in (B) for the experiments with different pressure conditions for 2 min at 4 °C. Measured values are represented by symbols with different shapes and colors (measurements from the same experiment have the same color and shape). Lines represent the predictions of the HEV inactivation model for the given holding time, where the line color correspond to environmental conditions of the same-colored measurements. For example in (A) the red circles represent measurements at 20 °C and 400 MPa while the red line gives the predictions by the model at the very same conditions from 0 to 10 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

probably indicating disassembled virus particles, as well as distorted particles, were found here.

4. Discussion

HPP is increasingly used for preservation of food. Here, the efficiency of this technique with regard to HEV inactivation was investigated. The cell culture-adapted genotype 3c strain 47832c was used with a titration system based of HEV detection using immunofluorescence. In Europe, genotype 3c is one of major types currently circulating (Abravanel et al., 2020), which has also been detected in food (Szabo et al., 2015). The system has been shown to be suitable for inactivation studies as shown for heat, salt and pH stability of HEV (Johne et al., 2016; Wolff et al., 2020a; Wolff et al., 2020b). Therefore, the same system was applied here for analysis of the stability of HEV in PBS at HPP. The use of PBS enables comparison with other treatments and viruses as several inactivation studies have been done using this matrix. The system enabled us to determine virus inactivation up to a 4 $\log_{10}$ reduction in virus titer, which is a value also used for defining virucidal activity of disinfectants in European standards (Steinmann, 2004).

The results of the study show that HEV can be inactivated by HPP, although high pressure/time combinations have to be used for efficient inactivation. HPP at 20 °C was a bit less efficient than at 4 °C. Temperature dependency of the HPP effects on virus inactivation has also been described for other viruses, e.g., for murine norovirus, which is more efficiently inactivated by HPP at 0 °C or 4 °C as compared to 20 °C (Huang et al., 2014; Lou et al., 2011). One explanation for this phenomenon might be an additional inactivating effect of freezing during pressure reduction at low temperatures. However, a higher degree of inactivation at lower temperature was also found at low pressures, where no freezing of samples was observed during pressure release.

By increasing the pressure or the holding time, the inactivating effect became higher. Overall, HEV showed a fairly high stability against HPP treatments. At a pressure of 400 MPa for 2 min, which corresponds to treatment conditions widely used in food industry, HEV infectivity was decreased for 1 $\log_{10}$ ffu/ml (20 °C) or 2 $\log_{10}$ ffu/ml (4 °C). Even at 600 MPa for 2 min, remaining infectious HEV could be detected in one out of three replicates, although with very low quantities around the detection limit. A direct comparison with data from other viruses is difficult as different matrices and temperature/pressure/time regimes are mostly applied in the published studies. Feline calicivirus, a surrogate for human norovirus, showed a >7 $\log_{10}$ infectivity decrease after treatment with 450 MPa at 15 °C for 1 min in cell culture medium as well as in mineral water (Buckow et al., 2008). Murine norovirus showed a >6 $\log_{10}$ infectivity decrease after treatment with 450 MPa at 20 °C for 5 min in cell culture medium (Kingsley et al., 2007). In contrast, the infectious titer of hepatitis A virus decreased only 2 $\log_{10}$ after treatment at 400 MPa at 20 °C for 5 min in cell culture medium containing 15 g/l salt (Grove et al., 2009). Similarly, human norovirus in oysters was not completely inactivated at 400 MPa at 25 °C for 5 min, but only at 600 MPa at these conditions (Leon et al., 2011). This indicates that human foodborne viruses like hepatitis A virus or human norovirus behave more similar like HEV regarding HPP inactivation, but even among these, HEV seems to have a higher stability to HPP.

Differences become obvious when comparing our results to that of a study recently published by Nasheri et al. (2020). Whereas we found a continuous decline of infectivity by increasing pressure and time, Nasheri et al. reported nearly the same inactivating effects at 400 MPa or 600 MPa and at 1 min or 5 min holding time. Also, only minimal infectivity decreases of 2 $\log_{10}$ in culture medium and 0.5 $\log_{10}$ in liver pate were determined in that study, even at 5 min treatment at 600 MPa. In contrast, we observed >3.5 $\log_{10}$ infectivity decrease at 2 min treatment at 600 MPa. It should be mentioned that the same strain of HEV has been used in both studies, thus excluding a strain-specific effect. One difference between both studies is the method used for titration of remaining infectivity after treatment. Whereas we used an established

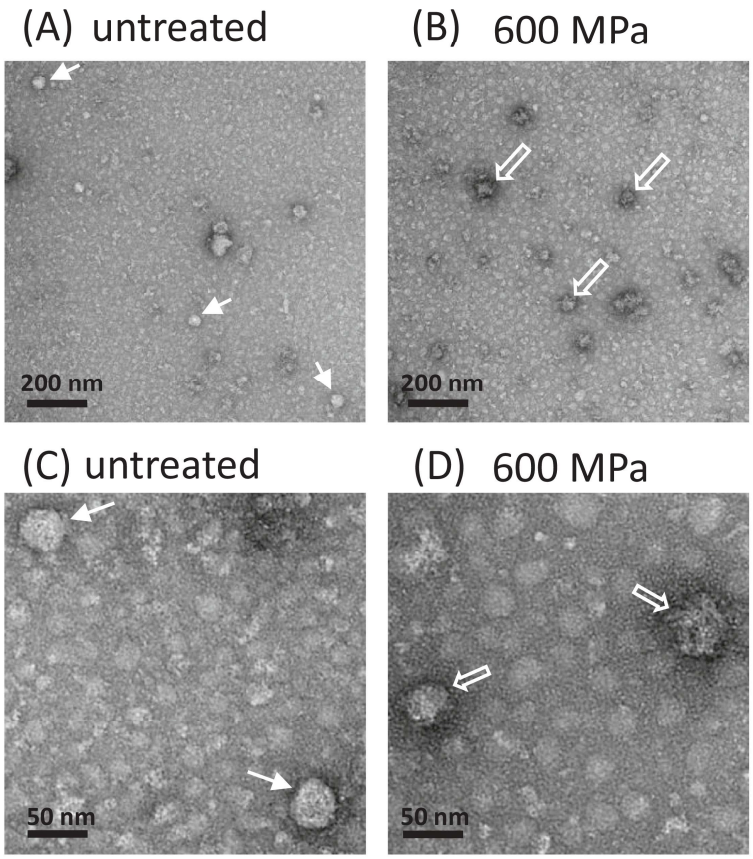

Fig. 4. Transmission electron microscopy analysis of HEV preparations. (A, C) untreated HEV, (B, D) HEV preparation treated at 600 MPa for 2 min at 20 °C. (A, B) lower magnification, (C, D) higher magnification. Small arrows: HEV-like particles, empty arrows: disassembled or distorted structures. Uranyl acetate staining.

immunofluorescence-based titration system, Nasheri et al. used a quantitative comparison of HEV-RNA at 2 weeks after inoculation of the samples onto cell cultures. It may be possible that remaining free RNA or RNA present in distorted non-infectious capsids of the inoculum have been measured in addition to RNA produced from infected cells by this method, which might led to an overestimation of HEV stability.

Another important difference between both studies is the use of different matrices as we used PBS and Nasheri et al. (2020) used culture medium and liver pate. Marked matrix-specific effects by HPP treatment have been described for other viruses (Kingsley, 2013). For example, murine norovirus showed a 7 $\log_{10}$ infectivity decrease after treatment with 400 MPa at 4 °C for 2 min in cell culture medium, whereas the same treatment in strawberry puree led to only 4 $\log_{10}$ infectivity decrease (Lou et al., 2011). Even in samples with only slightly different matrix composition, significant differences in inactivation by HPP have been described, e.g. for hepatitis A virus, which was more efficiently inactivated in marinated shellfish as compared to non-marinated shellfish (Pavoni et al., 2015), or for murine norovirus, which was markedly more resistant against HPP inactivation on dried berries as compared to fresh berries (Huang et al., 2014). Data on virus inactivation by HPP of meat products are scarce, but treatment of feline calicivirus with 400 MPa at 12 °C for 5 min on porcine liver resulted in 5 $\log_{10}$ infectivity decrease, whereas only a 1.3 $\log_{10}$ infectivity decrease was determined at the same conditions on ham (Emmoth et al., 2017).

Our data showed a nearly complete inactivation of HEV in PBS at the very high pressure of 600 MPa, which is also supported by our electron microscopic analysis. Small round structures with a typical shape of HEV particles were detected in the untreated samples, but not in those treated at 600 MPa for 2 min, where only structures resembling distorted and disassembled particles could be identified. Similar structural effects of HPP have been observed for other viruses, e.g., for murine norovirus or rotavirus (Araud et al., 2015; Lou et al., 2011).

Finally, the data could be used to set up a predictive model for HEV inactivation under various pressure (0.1–600 MPa) and holding time (0–10 min) conditions. As the model is empirical by nature, it should only be used within the range of the experimental pressure, temperature (4–20 °C) and holding time conditions. Further, it would be beneficial to generate independent experimental data to allow a validation of the model in future, which was beyond the scope of this study. As the generated model is provided in a software-independent model exchange format (PMF-ML) such a validation could now be performed by any other researcher as well.

Our study has certain limitations. First of all, only a small sample number could be investigated in a limited range of pressure/time combinations. Development of more efficient and easy to handle cell culture systems should be aimed in future in order to overcome the restrictions in sample numbers. Second, only treatment in PBS has been analyzed so far. The distinct effect of protein and other substances should be determined in future. Ideally, the HPP stability of HEV should be analyzed directly in meat products. However, at least in our laboratory, recovery of infectious HEV from meat products showed a very low efficiency (data not shown), thus preventing titration of HEV infectivity directly from those food matrices. Therefore, future studies should focus on the development of efficient methods for recovery of infectious HEV

from meat products.

5. Conclusions

HEV can be inactivated by HPP and a gradual decrease of infectivity is observed by higher pressure and longer holding time intervals. Compared to other viruses, HEV shows a high stability against HPP. A mathematical model has been generated allowing the prediction of HEV inactivation in PBS at distinct pressure/holding time combinations. In future, the generated data and the model should be validated, when efficient and reliable methods for analysis of HEV infectivity directly in meat products are available. Generally, it can be concluded from the study that HPP can be considered as a treatment method for food in order to decrease the risk of foodborne HEV transmission. However, the efficiency of its application has to be assessed in future by considering any possible effects of the specific matrix, the expected amount of HEV in the meat preparation and the desired HEV concentration in the final product.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.109013.

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4.5 Publication 4: Stability of Hepatitis E Virus After Drying on Different Surfaces

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ORIGINAL PAPER

Stability of Hepatitis E Virus After Drying on Different Surfaces

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Abstract
The hepatitis E virus (HEV) causes acute and chronic hepatitis in humans. The zoonotic HEV genotype 3 is mainly transmitted by consumption of contaminated food produced from infected animals. However, transmission via contaminated surfaces has also to be considered. Here, the genotype 3c strain 47832c was dried on steel, wood, plastics and ceramics, stored at 23 °C or 3 °C for up to 8 weeks and remaining infectivity was titrated on cell culture. During the drying process, only a mean 0.2 $\log_{10}$ decrease of HEV infectivity was observed. At 23 °C, remaining infectious virus was detected until week 4 on most surfaces, but HEV was completely inactivated (> 4 $\log_{10}$ decrease) after 8 weeks. At 3 °C, HEV was detectable up to 8 weeks on most surfaces, with an average 2.3 $\log_{10}$ decrease. HEV showed the highest stability on plastics, which was lower on ceramics and steel, and lowest on wood. The addition of bovine serum albumin mimicking high protein load had only a slight stabilizing effect. In conclusion, HEV shows a high stability against drying and subsequent storage on different surfaces. Strict application of hygienic measures during food production is therefore crucial in order to prevent HEV persistence on surfaces and subsequent cross-contamination.

Keywords Hepatitis E virus · Drying · Stability · Inactivation · Surfaces

Introduction

The hepatitis E virus (HEV) is an important agent of human hepatitis. Large outbreaks of hepatitis E were reported from developing countries, whereas sporadic cases are predominant in industrialized countries (Goel & Aggarwal, 2020; Webb & Dalton, 2019). In the last years, increasing numbers of hepatitis E cases have been notified in Europe (Aspinall et al., 2017). The disease is mainly characterized by acute hepatitis. However, chronic HEV infections, which can lead to life-threatening liver cirrhosis, are increasingly described in immunosuppressed transplant patients (Narayanan et al., 2019). In addition, extrahepatic manifestations like neurologic disorders have been attributed to HEV infection (Velavan et al., 2021).

HEV belongs to the family *Hepeviridae*, which is characterized by small single-stranded RNA viruses (Purdy et al., 2017). Two different particle forms are known for HEV: non-enveloped particles with a diameter of ~ 30 nm and quasi-enveloped particles with a diameter of ~ 40 nm (Nagashima et al., 2017). Whereas non-enveloped particles are mainly found in feces, quasi-enveloped particles exist in serum and cell culture supernatant (Wolff et al., 2020a; Yin et al., 2016a). Both particle forms are infectious in cell culture, with a higher infectivity of the non-enveloped particles compared to the enveloped particles (Capelli et al., 2020; Yin et al., 2016b).

Most of the human-pathogenic HEV strains are grouped into genotypes HEV-1 to HEV-4. HEV-1 and HEV-2 exclusively infect humans and their major route of transmission is via fecally contaminated drinking water (Pallerla et al., 2020). In contrast, HEV-3 and HEV-4 are zoonotic and circulate in reservoir animals such as wild boars and pigs (Pavio et al., 2017). The main transmission route of these genotypes is foodborne by consumption of undercooked meat or raw meat products from infected animals. Especially, raw liver and sausages containing raw liver have been linked to hepatitis E outbreaks in the past (Colson et al., 2010; Masuda et al., 2005; Matsuda et al., 2003). Furthermore, RNA of HEV was frequently detected in different meat products derived from domestic pigs, wild boars or deer (Pavio et al., 2017; Szabo et al., 2015).

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The distinct stability of HEV in meat and meat products is mainly unknown because no reliable method for direct infectivity measurement of HEV in these matrices exists so far (Cook et al., 2017). Cell culture systems assessing the infectivity of HEV in solutions have been improved recently (Capelli et al., 2020; Meister et al., 2019; Schemmerer et al., 2016, 2019; Todt et al., 2020), but are still laborious and time-consuming compared to many other viruses. Recent stability studies using the cell culture-adapted HEV strain 47832c in phosphate-buffered saline (PBS) showed a very high resistance of HEV against a broad pH range and high salt concentrations (Wolff et al., 2020a, 2020b). Conditions prevailing in raw sausage production could not completely inactivate HEV, indicating that infectious virus can be expected in raw sausages if sufficiently contaminated starting material was used (Wolff et al., 2020a, 2020b). Another scenario of food contamination could involve cross-contamination via surfaces used during food production and preparation. However, no data on stability of HEV after drying on surfaces are available so far, making an assessment of the risk of HEV transmission through this pathway difficult.

In this study, the stability of HEV after drying on different surfaces was assessed, under conditions simulating those at food production and storage. Using a cell culture-based system for titration of HEV infectivity, the decrease of infectivity was analyzed directly after drying and after storage at 23 °C or 3 °C for up to 8 weeks. The results should help to estimate the risk of HEV transmission by cross-contamination during food production and through contaminated surfaces in general.

Materials and Methods

Virus and Cells

For all experiments, the HEV genotype 3c strain 47832c (GenBank acc. no. KC618403) was used. This strain was originally isolated from a serum sample of a chronically infected transplant patient (Johne et al., 2014). For production of a virus stock, an A549 cell line persistently infected with this virus strain (Johne et al., 2014, 2016) was cultivated. For infectivity titrations, the cell line A549/D3, a subclone of cell line A549 showing enhanced susceptibility to HEV strain 47832c (Johne et al., 2016; Schemmerer et al., 2016), was used.

Preparation of Virus Stock

Virus stock preparation was performed as described (Wolff et al., 2020a). Briefly, the persistently HEV-infected A549 cell line was cultivated in a humidified incubator for 7 days and thereafter split 1:2 for culture expansion. For virus harvest, cells were subjected to a triple freeze/thaw cycle and the supernatant was collected and stored at − 20 °C until further use. Thereafter, the harvested supernatants were pooled, cell debris were removed by low-speed centrifugation and virus particles were concentrated via ultracentrifugation (Wolff et al., 2020a). The resulting pellets were redispersed in phosphate-buffered saline (PBS, PAN-Biotech GmbH, Germany) with 1/10 or 1/100 volume (depending on initial virus concentration) as compared to the original supernatant volume. After an additional cleaning step by centrifugation, the virus concentrates were combined, mixed, aliquoted and stored at − 20 °C. The resulting HEV stock dispersion had an infectivity of 2.8×10^4 focus-forming units (ffu)/ml.

Virus Stability Testing After Drying on Surfaces

A stock solution of bovine serum albumin (BSA), was prepared by dissolving 1.65 g BSA (Cell Signaling Technology, USA) in sterile PBS up to a total volume of 50 ml. The BSA solution was thereafter sterile filtrated (PES membrane, 0.22 µm, Merck Millipore Ltd., USA), aliquoted and stored at − 20 °C. To prepare an HEV stock preparation with BSA, 4 ml of the HEV stock dispersion was mixed with 0.4 ml of the BSA stock solution, leading to a final BSA concentration of 3 g/l, as suggested for virus inactivation studies on non-porous surfaces (Rabenau et al., 2012). To prepare a virus stock preparation without BSA, 4 ml of the HEV stock dispersion was mixed with 0.4 ml sterile PBS. The resulting virus titer of the preparations (with and without BSA) was 2.5×10^4 ffu/ml.

Four different surface types were selected based on their common use in food production and during food preparation: steel (a common surface material in slaughter houses, cutting plants or groceries), wood and plastics (common material of cutting boards), and ceramics (commonly used for dishes). For contamination experiments, the sample carriers consisting of circular steel plates (stainless steel X2CrNi18-9, surface 2B, YC INOX CO., LTD., Taiwan), wood boards (European beech, Continenta GmbH, Germany), plastics boards (polyethylene, IKEA, Sweden) and spot plates of ceramics (glazed porcelain, Roth, Germany) were placed under a sterile workbench. Thereafter, 275 µl aliquots of the corresponding virus stock with or without BSA were placed on marked sites of the sample carrier of the corresponding material. Drying was done at room temperature. After 1 h, which resulted in a 2- to 3-fold volume reduction without complete drying, two aliquots were aspirated from each surface material with or without BSA using a pipette, put into a 1.5 ml tube and filled up to 0.5 ml with sterile PBS. Virus left on the surface was then picked up with a sterile PBS-moistened cotton swab (Boettger GmbH & Co. KG, Germany), which was placed into the tube containing the corresponding aspirated virus aliquots (sample “before

drying"). Each tube was vortexed for 1 min, and the cotton swab was squeezed and removed from the tube. All tubes were centrifuged at 2000×*g* for 10 min at 4 °C and 450 µl of each supernatant was transferred into a new tube and stored at 4 °C until virus titration, which was performed at the same day. When the drying process was complete, the dried aliquots from each surface material were picked up with sterile PBS-moistened cotton swabs, placed into 1.5 ml tubes containing 0.5 ml sterile PBS and processed as described above (sample "after drying", t_0). For storage experiments, the sample carriers were removed from the bench after complete drying and immediately placed into a plastic box together with a data logger (OM-24, OMEGA, USA) recording temperature and relative humidity (RH) every hour. For the 23 °C experiment, the closed box was stored in the dark at room temperature. For the 3 °C experiment, two open water bowls (diameter: 20 cm) filled with aqua bidest. were placed within the box beside the sample carriers in order to maintain high humidity. After adding the data logger, the box was closed and stored in a dark cooling room. Samples were taken by swabbing as described above at 1 day, 1 week, 4 weeks and 8 weeks after contamination. All experiments were performed with two biological replicates.

Titration of HEV Infectivity

HEV infectivity titrations were performed as described (Johne et al., 2016). Briefly, confluent A549/D3 cell layers were infected with tenfold dilution series of the samples in a 96-well plate format. Each of the two biological replicates derived from a specific experimental condition (see above) was titrated in four technical replicates, resulting in eight titrated subsamples for each experimental condition. After infection, the cells were incubated for 2 weeks and subsequently stained by immunofluorescence with an HEV capsid protein-specific rabbit antiserum and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Fluorescent cell foci were manually counted using a confocal fluorescence microscope (Opera Phenix, PerkinElmer). A focus was defined as at least 2 contiguous cells showing a clear intraplasmatic fluorescence. The resulting foci numbers were multiplied by the dilution factors of the corresponding wells, in order to calculate ffu/ml, which were $\log_{10}$-transformed. Arithmetic means from the eight titrated subsamples of each distinct experimental condition and the respective standard deviations were calculated using MS Excel.

Data Analysis

All three experiments were analyzed according to the differences between the conditions. As a prerequisite, all experimental results were tested for normal distribution using the Shapiro–Wilk test and *q*–*q* plots. As most results did not show a normal distribution, the Kruskal–Wallis test was used to analyze the general differences between all conditions for each experiment. In case of a significant result a pairwise comparison of the conditions was performed using the Wilcoxon test for unpaired samples, to identify respective significantly different pairs. For all statistical tests performed, a p value < 0.05 was defined as significant. All statistical tests were conducted with the software R 3.6.1 (R Core Team, 2020). For the descriptive analyses and the plots, the software MS Excel was used.

Results

Effect of Drying on HEV Infectivity

The effect of drying on HEV infectivity was analyzed using four different surface materials and the presence or absence of BSA mimicking high protein load. HEV aliquots were added to the surface materials and the infectivity before and immediately after drying was assessed. The mean temperature during the drying process was 22 °C. As shown in Fig. 1, only minor differences in the mean HEV infectivity before and after drying are evident. In line with this, statistical analyses indicated no significant differences within all analyzed condition pairs. However, direct comparison of mean values might indicate a general trend of slightly decreased infectivity after drying.

In detail, the HEV mean infectivity titers decreased after drying for almost all conditions tested, in the range from 0.1 $\log_{10}$ ffu/ml for plastics with BSA to 0.7 $\log_{10}$ ffu/ml for wood without BSA. Only for steel with BSA, an increase in mean infectivity of 0.5 $\log_{10}$ ffu/ml was determined after drying; however, with overlapping standard deviations. The calculated arithmetic mean of the decrease after drying with and without BSA on all surfaces was 0.2 $\log_{10}$ ffu/ml.

By comparing the differences with and without BSA for each material, a general trend of slightly decreased infectivity without BSA as compared to the condition with BSA is likely. These mean differences range from 0.3 $\log_{10}$ ffu/ml for plastics and ceramics to 0.4 $\log_{10}$ ffu/ml for wood. Steel showed a mean difference of 0.6 $\log_{10}$ ffu/ml, including the mentioned increase of infectivity after drying with BSA.

By comparing different materials in case of the absence of BSA, steel showed the smallest inactivation effect with 0.1 $\log_{10}$ ffu/ml, followed by plastics with 0.3 $\log_{10}$ ffu/ml and ceramics with 0.6 $\log_{10}$ ffu/ml, whereas wood showed the strongest inactivation effect with 0.7 $\log_{10}$ ffu/ml. In the presence of BSA, steel showed the least inactivation effect of HEV with − 0.5 $\log_{10}$ ffu/ml, followed by plastics with 0.1 $\log_{10}$ ffu/ml, and the strongest inactivation effect was found by ceramics and wood with 0.3 $\log_{10}$ ffu/ml.

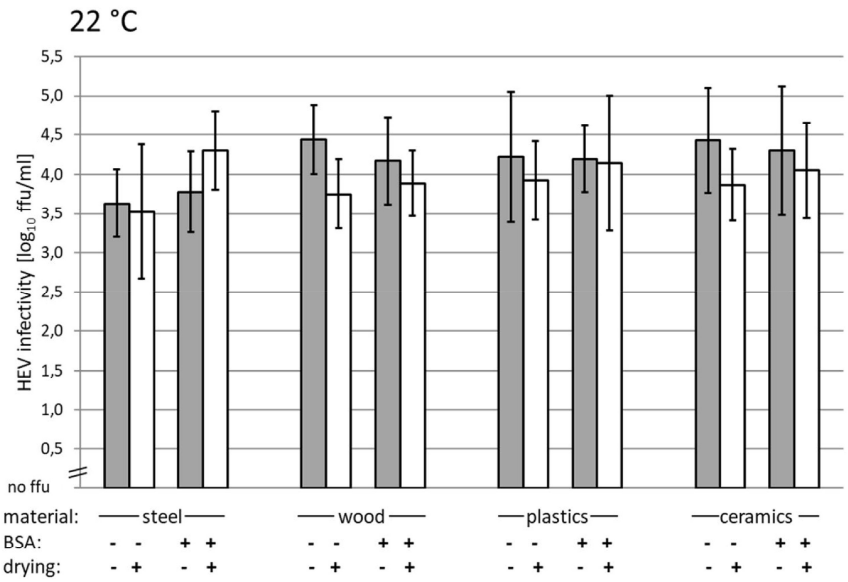

Fig. 1 Stability of HEV against drying at 22 °C on different surfaces. The paired columns show infectivity before (gray) and after (white) drying. Each pair of columns represents a specific surface material in the presence or absence of bovine serum albumin (BSA) as loading substance. The arithmetic mean of two replicates, which were titrated in 4 replicates each, is shown. Scaled in $\log_{10}$ focus-forming units (ffu)/ml. Error bars indicate the standard deviations

Long-Term Stability of Dried HEV on Different Surfaces at 23 °C

In order to investigate the long-term stability of dried HEV at ambient conditions usually present in groceries or kitchens, a storage experiment for up to 8 weeks at room temperature and low RH was performed. Briefly, HEV with and without BSA was dried on four different surface materials and stored in a plastic box in the dark. The recorded data for temperature and RH are shown in Supplementary Data S1, indicating for the whole experiment arithmetic means of 23 °C and 26% RH. The results generated without BSA (Fig. 2A) and with BSA (Fig. 2B) indicate a continuous decline of infectivity, with minor differences between the surface materials.

Whereas the mean values at t_0 (immediately after drying) and after one day storage were nearly identical, an almost linear decrease of mean infectivity was evident from 1 day to 4 weeks storage for all materials without BSA (Fig. 2A). The mean infectivity on wood declined to no infectivity (3.8 $\log_{10}$ ffu/ml decrease) after 4 weeks, whereas residual infectivity was detected at this time-point on the other surfaces, with mean infectivity decreases of 3.1 $\log_{10}$ ffu/ml for ceramics, 3.0 $\log_{10}$ ffu/ml for plastics and 3.2 $\log_{10}$ ffu/ml for steel. After 8 weeks, no residual infectivity could be detected for all materials.

The diagram in Fig. 2B, which shows the results for the experiment with BSA addition, is largely similar to that in Fig. 2A. Again, no residual infectivity was detected on all surfaces after 8 weeks. After 4 weeks, residual infectivities were determined on wood (2.8 $\log_{10}$ ffu/ml decrease), ceramics (4.0 $\log_{10}$ ffu/ml decrease) and plastics (3.9 $\log_{10}$ ffu/ml decrease), whereas no infectivity could be detected on steel at this time-point.

Long-Term Stability of Dried HEV on Different Surfaces at 3 °C

In order to investigate the long-term stability of dried HEV at low temperature conditions usually present in refrigerators and cooling facilities, a storage experiment for 8 weeks in a cooling room at high RH was performed. Briefly, HEV with and without BSA was dried on four different surface materials and stored together with open water reservoirs in a plastic box placed in a cooling room in the dark. The recorded data for temperature and RH are shown in Supplementary Data S2, indicating for the whole experiment arithmetic means of 3 °C and 98% RH. After 40 days, the box with the samples had to be transferred to another cooling room, which resulted in a slight temperature increase of about 3 °C (Supplementary Data S2). The results generated without BSA (Fig. 3A) and with BSA (Fig. 3B) indicate a trend of a lower decline of infectivity as compared to the storage at 23 °C, with more obvious differences between the surface materials.

The data from the experiment without BSA (Fig. 3A) indicated similar mean infectivity values for t_0 and one day of storage. Whereas for plastics only a slight mean decrease of about 1.0 $\log_{10}$ ffu/ml was observed within 4 weeks, the values for the other materials dropped more pronounced: for ceramics (2.6 $\log_{10}$ ffu/ml decrease), steel (3.1 $\log_{10}$ ffu/ml decrease) and wood (3.5 $\log_{10}$ ffu/ml decrease). Between weeks 4 and 8, the mean decrease of infectivity for plastics

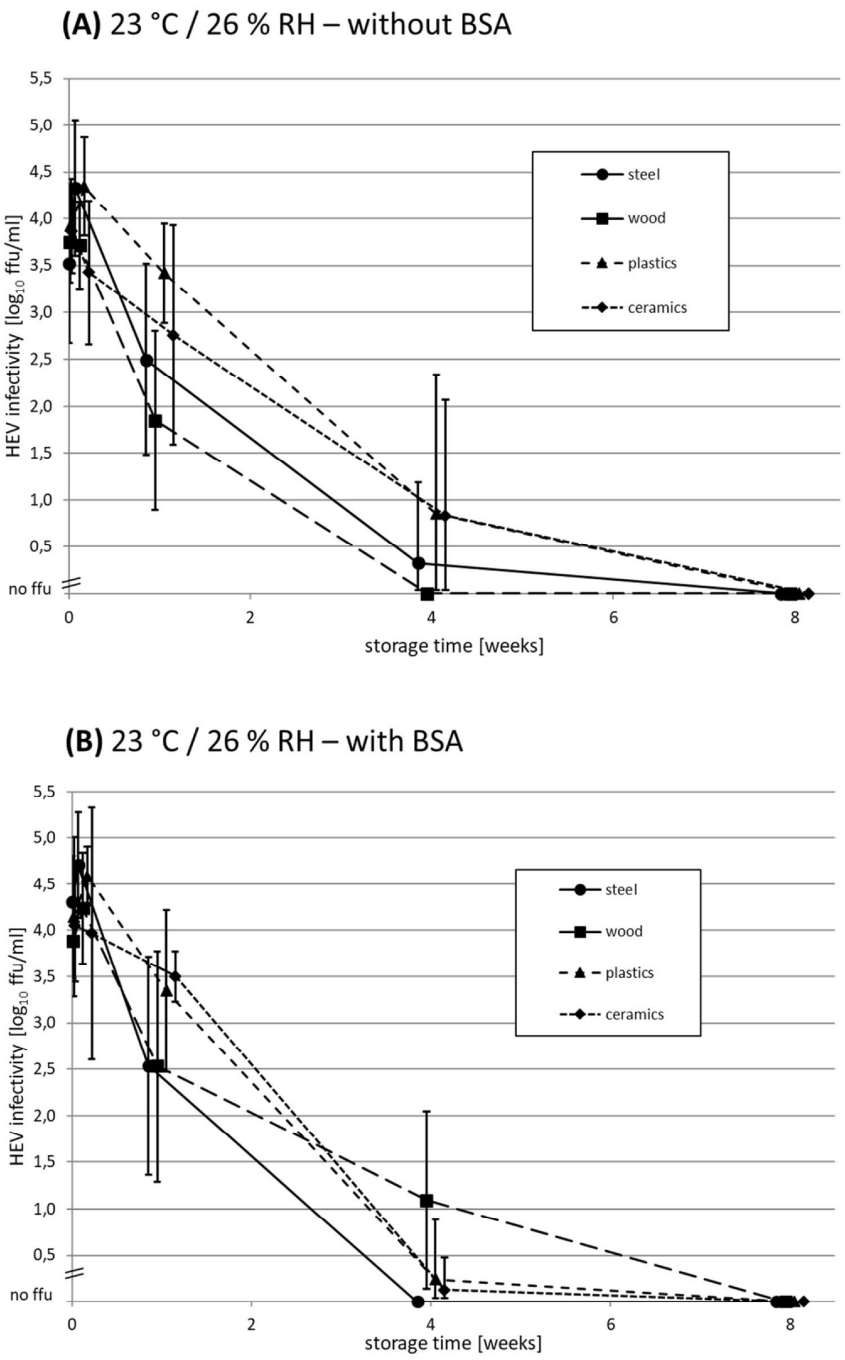

Fig. 2 Time-course analysis of HEV infectivity after drying on different surface materials at 23 °C and 26% relative humidity (RH) for 8 weeks **A** without and **B** with adding of bovine serum albumin (BSA) as loading substance. Each data point represents the mean HEV infectivity on a specific surface material at the indicated timepoint of storage. The arithmetic mean of two replicates, which were titrated in 4 replicates each, is shown. Scaled in $\log_{10}$ focus-forming units (ffu)/ml. Error bars indicate the standard deviations

was minimal with 0.2 $\log_{10}$ ffu/ml, whereas no residual infectivity (> 3.5 $\log_{10}$ ffu/ml decrease) could be detected after 8 weeks storage for steel and wood. Ceramics showed a mean increase of infectivity of 1.2 $\log_{10}$ ffu/ml between 4 and 8 weeks; however, with large standard deviations especially at 4 weeks of storage. By comparing the inactivation curves shown in Fig. 3A, HEV shows a trend of the highest stability on plastics, followed by ceramics and steel, whereas HEV stability was lowest on wood.

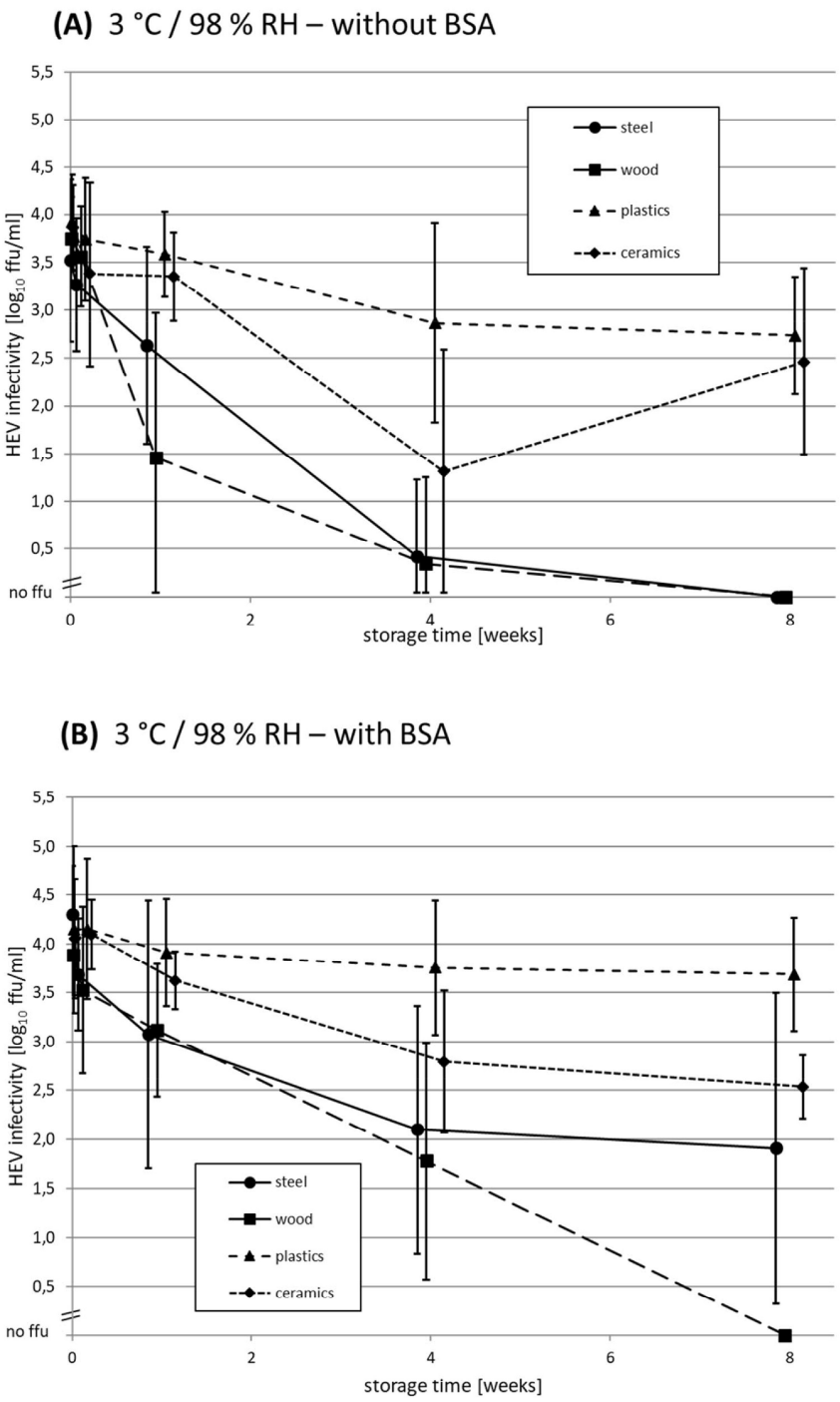

Fig. 3 Time-course analysis of HEV infectivity after drying on different surface materials at 3 °C and 98% relative humidity (RH) for 8 weeks **A** without and **B** with adding of bovine serum albumin (BSA) as loading substance. Each data point represents the mean HEV infectivity on a specific surface material at the indicated timepoint of storage. The arithmetic mean of two replicates, which were titrated in 4 replicates each, is shown. Scaled in $\log_{10}$ focus-forming units (ffu)/ml. Error bars indicate the standard deviations

By analyzing the data for the conditions with added BSA (Fig. 3B), no differences of mean infectivity between t_0 and one day of storage was observed for plastics and ceramics, whereas a mean decrease of infectivity of about 0.5 $\log_{10}$ ffu/ml was detected for steel and wood. During storage for 8 weeks, HEV infectivity on plastics showed a mean decrease of only 0.4 $\log_{10}$ ffu/ml. At the same time, the mean HEV infectivity decreased on ceramics by 1.6 $\log_{10}$ ffu/ml, and on steel by 2.4 $\log_{10}$ ffu/ml. The largest mean decrease of infectivity showed HEV on wood with 3.9 $\log_{10}$ ffu/ml after 8 weeks of storage. The HEV inactivation curves shown in Fig. 3B indicate the trend that HEV was most stable on plastics, followed by ceramics and steel, and had the lowest stability on wood, under these conditions.

Statistical Analyses

The result of statistical analysis for the first experiment (effect of drying) is described in chapter 3.1., indicating no significant differences between the analyzed conditions. The p values calculated from the storage experiments are summarized in Table 1.

As evident from Table 1, the frequency of significant differences between t_0 and the other time-points (bold p values) increases with longer time intervals, indicating a time-dependent inactivation of HEV. In addition, more significant differences were found at 23 °C as compared to 3 °C, indicating faster inactivation at higher temperature. Only minor differences between the numbers of significant values derived with or without adding BSA are evident, indicating only a low effect of adding BSA. When comparing the surface materials, wood showed the highest number of significant differences between t_0 and the other time-points, followed by steel and ceramics, whereas plastics showed the lowest number. This indicates that HEV inactivation is fastest on wood, followed by steel and ceramics, and slowest on plastics.

Discussion

Knowledge about the stability of viruses under different environmental conditions is essential to uncover their transmission pathways, to develop concepts for prevention of virus transmission and to establish effective methods for virus inactivation. Although the assessment of HEV stability is still hampered by the lack of rapid and easy-to-perform methods for HEV infectivity determination, significant progress has been made in the evaluation of the stability of HEV against various physico-chemical treatments recently. Generally, a high stability of HEV against pH, salts, chlorine, UV light and high hydrostatic pressure was assessed in previous studies (Girones et al., 2014; Guerrero-Latorre et al., 2016; Imagawa et al., 2018; Johne et al., 2021; Wolff et al., 2020a, 2020b), which is in line with the major transmission pathways of HEV through contaminated food and water. In contrast, the stability of HEV after drying on surfaces is not known so far. However, contaminated surfaces may be involved in HEV transmission, e.g., by cross-contamination of food or by direct contact resulting in smear infections.

Table 1 Statistical analyses of data from the experiments investigating storage of dried HEV on different surfaces

Conditions	t_0 vs. 1 day	t_0 vs. 1 week	t_0 vs. 4 week	t_0 vs. 8 week
Plastics, without BSA, 23 °C	0.183	0.570	**0.009**	**0.002**
Ceramics, without BSA, 23 °C	0.346	**0.037**	**0.004**	**0.002**
Steel, without BSA, 23 °C	0.167	0.115	**0.003**	**0.002**
Wood, without BSA, 23 °C	0.869	**0.003**	**0.002**	**0.002**
Plastics, with BSA, 23 °C	0.103	0.200	**0.002**	**0.002**
Ceramics, with BSA, 23 °C	0.776	0.126	**0.002**	**0.002**
Steel, with BSA, 23 °C	0.254	**0.007**	**0.002**	**0.002**
Wood, with BSA, 23 °C	0.223	**0.033**	**0.003**	**0.002**
Plastics, without BSA, 3 °C	0.803	0.296	0.058	**0.011**
Ceramics, without BSA, 3 °C	0.460	0.115	**0.004**	**0.004**
Steel, without BSA, 3 °C	0.734	0.083	**0.003**	**0.002**
Wood, without BSA, 3 °C	0.427	**0.008**	**0.002**	**0.002**
Plastics, with BSA, 3 °C	0.803	0.633	0.699	0.306
Ceramics, with BSA, 3 °C	1.000	0.234	**0.020**	**0.003**
Steel, with BSA, 3 °C	0.103	0.183	**0.004**	**0.006**
Wood, with BSA, 3 °C	0.356	0.059	**0.003**	**0.002**

Time point t_0 was compared with all other time-points for all conditions. The calculation methods used are described in detail in chapter 2.5. Results are presented as p values. Bold p values indicate significant differences ($p < 0.05$)

To investigate the HEV stability after drying, a cell culture system using the cell culture-adapted HEV subtype 3c strain 47832c was used (Johne et al., 2014). Subtype 3c represents one of the predominant subtypes detected in humans and animals in Europe (Adlhoch et al., 2016; Anheyer-Behmenburg et al., 2017). In addition, this cell culture system was used previously in several studies assessing the stability of strain 47832c (Johne et al., 2016, 2021; Wolff et al., 2020a, 2020b), enabling direct comparison of the data. In our study, the cell culture system enabled an initial assessment of HEV stability after drying on surfaces. However, as the method is still laborious and time-consuming, only a limited number of conditions and replicates could be investigated. Future studies using improved methods are therefore desirable in order to broaden the conditions to other surface materials and temperatures, and to gain more precise data regarding variation of mean errors.

In the tested preparation, both non-enveloped and quasi-enveloped particles are present (Wolff et al., 2020a). It has been shown that non-enveloped particles mainly occur in feces and quasi-enveloped particles in serum of patients, although non-enveloped HEV particles have also been identified in human sera in a recent study (Costafreda et al., 2021). The particle form present in pig liver, meat and meat products is unknown so far. However, the presence of a mixture of enveloped particles (budded from cells or originating from residual serum in the meat) and non-enveloped particles (released from damaged cells or generated by removal of the envelope by environmental factors) is most probable. Therefore, particle mixtures similar to that used in our experiments may reflect those occurring in meat in practice, although the distinct combination of particle forms in certain meat products may vary. A higher infectivity of non-enveloped particles as compared to quasi-enveloped particles has been described using cell culture studies (Capelli et al., 2020; Yin et al., 2016b), and a removal of the envelope due to environmental factors may therefore increase infectivity. In order to analyze the distinct contribution of inactivating and infectivity-increasing processes during drying and storage, additional experiments with preparations of separated non-enveloped and enveloped particles should be performed in future studies.

The resistance of viruses against the drying process represents the major factor for their survival in dried condition on surfaces (Sánchez & Bosch, 2016). In our experiments, only slight decreases of HEV infectivity have been found during the drying process, with no statistical significance between the titers before and after drying. This indicates that HEV is highly stable against drying, and the persistence of infectious virus has to be expected on surfaces after contact to contaminated meat or to excretions from infected animals or humans. The high stability was evident for all tested surface types and the addition of BSA, which was used as loading substance, had only a minor stabilizing effect. Other enterically transmitted viruses like hepatitis A virus (HAV), norovirus, rotavirus or astrovirus have also been shown to be highly stable against the drying process (Mahl & Sadler, 1975; Keswick et al., 1983; Sattar et al., 1986; Sobsey et al., 1988; Abad et al., 1994; Abad et al., 2001). By comparison of HAV, rotavirus, poliovirus and adenovirus during drying on different smooth surfaces, HAV showed the highest stability (Abad et al., 1994). According to the results of our study, HEV inactivation during drying showed similar characteristics as described for HAV. For example, in the absence of a loading substance during drying on ceramics, the mean infectivity was reduced by 0.5 $\log_{10}$ for HAV (Abad et al., 1994) and by 0.6 $\log_{10}$ for HEV. In the presence of a loading substance, the mean infectivity during drying on ceramics decreased by 0.5 $\log_{10}$ for HAV (Abad et al., 1994) and by 0.3 $\log_{10}$ for HEV.

The persistence of infectious HEV after drying on different surfaces and subsequent storage was assessed in our study at two different conditions, which simulated typical scenarios during food production and preparation. First, ambient conditions usually present in groceries or kitchens (or in hospitals, or generally in rooms) with a temperature of 23 °C and a low RH of 26% were tested. Second, low temperature conditions usually present in refrigerators and cooling facilities with a temperature of 3 °C and a high RH of 98% were chosen. This approach enabled the testing of typical scenarios, but the distinct contributions of temperature and RH to HEV inactivation could not be differentiated and should therefore be analyzed in future experiments. Generally, stability of HEV was lower at the ambient conditions compared to the low temperature conditions. Whereas at ambient conditions, HEV infectivity was mostly destroyed after 4 weeks and totally absent after 8 weeks, remaining infectious HEV could be detected at 8 weeks in 5/8 samples at low temperature conditions. Higher stability at 4 °C as compared to 20 °C has also been described for HAV, poliovirus and adenovirus after drying on surfaces (Abad et al., 1994), although the effect was less pronounced than for HEV.

Especially at the low temperature condition, a marked effect of the distinct surface material was observed in our study. Here, HEV showed an exceptionally high stability on plastics, with only 0.4 $\log_{10}$ ffu/ml infectivity decrease after 8 weeks in the experiment with addition of BSA. Ceramics and steel showed moderate inactivation rates, and HEV was almost completely inactivated (3.9 $\log_{10}$ ffu/ml decrease) on wood under the same conditions. The reasons for the differences are not known, but it could be speculated that the porous surface of wood absorbs water from the virus particles with higher efficiency as compared to non-porous surfaces, which may lead to a faster virus inactivation, as recently hypothesized for SARS-CoV-2 (Corpet, 2021). This

is in line with results from experiments with HAV, showing a higher stability on smooth surfaces (aluminium, ceramics) as compared to porous surfaces (paper) (Abad et al., 1994). Generally, the stability of HEV and HAV at low temperature and high RH turns out to be rather similar, at least for surface materials where data are available for both viruses. For example, in the presence of a loading substance after drying on ceramics for 8 weeks, the mean infectivity was reduced by 1.1 $\log_{10}$ for HAV (Abad et al., 1994) and by 1.6 $\log_{10}$ for HEV, and on metal by 1.5 $\log_{10}$ for HAV (aluminium) (Abad et al., 1994) and by 2.4 $\log_{10}$ for HEV (steel).

Our study has some limitations. Because of the necessary use of the laborious and time-consuming titration system, only a low number of samples could be analyzed, thus limiting the analyzed conditions and replications of the experiments. As already mentioned, the distinct influence of temperature and humidity could not be differentiated because of the specifically selected experimental conditions. Also, a discrimination of the inactivation profile of non-enveloped vs. quasi-enveloped particles could not be assessed. In addition, only one strain has been investigated and other strains may show different behaviors. The use of PBS and addition of BSA may not completely reflect the complex composition of blood, feces or meat juice, which are suspected to be the most probable matrices containing HEV in the field. Generally, the transmission rate of HEV to surfaces and from the surfaces to food and humans has to be determined in the future to better assess the transmission probability under field conditions.

It can be concluded that HEV is highly resistant against the process of drying and shows a high stability against long-term storage on several surfaces. The highest stability was determined at low temperature and high RH resulting in detection of infectious virus for as long as 8 weeks in most cases. Therefore, remaining infectious virus has to be expected for long time on surfaces initially contaminated with HEV. Subsequent virus transmission to food by contact to the contaminated surfaces or direct virus transmission to humans by smear infections should therefore be considered. Although the distinct risk of human infection via these pathways cannot be assessed, because the minimal infectious dose for oral infection of humans is not known so far, preventive measures should be taken to minimize the transmission risk. This should include strict application of hygienic measures during food production to prevent cross-contamination of other food. Selection of suitable surface materials used during food preparation may also support hygienic measures as the type of surface material has been shown to have significant effects on HEV stability. Further studies should focus on testing of the HEV drying stability directly in biological matrices and include the analysis of transmission rates of HEV to and from surfaces to enable a more complete risk assessment on HEV transmission via surfaces.

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Data Availability Data are available in Supplementary Materials and additional data can be retrieved upon request by R.J. (Reimar.Johne@bfr.bund.de).

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5 Discussion and perspectives

5.1 Selection of methods for infectivity determination of HEV

In order to perform the stability and inactivation studies as presented in this thesis, the availability of a suitable method for infectivity determination of HEV was an important prerequisite. However, as already described in the introduction, the methods for infectivity determination of HEV are severely limited so far. Before starting the experiments, advantages and disadvantages of available systems have been considered.

PCR-based systems are comparably easy to perform and well established for HEV. However, as they only detect the viral genome, they cannot directly distinguish between infectious and inactivated virus. Therefore, several modifications of PCR-based systems - so called “Viability PCR” - have been developed, which aim to detect only genomes, which are packaged into intact virus capsids. This includes systems using intercalating dyes, which prevent PCR amplification if they directly come in contact with the genome that is not packaged, or RNAse-based systems, which degrade the genome if not packaged. Those systems have been shown to somewhat correlate with methods directly measuring HEV infectivity [207]–[209]. However, they are still not fully developed, as they also detect genomes that are packaged in capsids, which are partly damaged and therefore no longer infectious, which results in an overestimation of infectivity. Although these methods are fast and inexpensive, they have therefore not been chosen for our studies.

Animal experiments have been previously used for infectivity determination of HEV using the well-established pig inoculation model [198], [199]. This method can provide profound evidence on the presence of infectious HEV in a sample. However, it cannot provide a quantitative statement, and it is costly and ethical concerns limit its broad use. Moreover, the number of samples that can be analyzed by this method is strictly limited, therefore decreasing the statistical significance and the number of tested conditions.

Infectivity determination by cell culture has therefore been established as the method of choice. Several HEV cell culture systems have been developed recently [192]. However, all of them are still inefficient as compared to those for many other viruses,

making their broad application difficult. For the studies presented here, an established cell culture system was chosen, which uses an HEV genotype 3c strain thus representing one of the major genotypes circulating in Germany [47], [204], [210]. This laboratory strain has been shown previously to contain a genome insertion, which leads to increased replication in cell culture [211]. Combined with an optimized cell line, infectivity can be determined robustly and quantitatively by counting foci of infected cells using immunofluorescence of viral proteins. The system has been previously shown to be suitable for infectivity determination in a study analyzing heat stability of HEV [204]. However, it is still laborious and time-consuming. Moreover, the system has still limitations regarding the amount of virus that can be produced for use in the inactivation experiments.

5.2 Cell culture system optimization and characterization of HEV stocks

To increase the amount of produced virus, adjustments of parameters that could have an effect on HEV replication during the growth of the persistently infected cell line were tested in a first approach. These included variation in temperature and fetal bovine serum (FBS) concentration during the incubation period. However, these changes could not contribute to an increase in viral titer in the cell culture supernatant (data not shown).

Since HEV does not show a cytopathic effect, attempts to release virus particles from inside of the cells to the cell culture supernatant were made next. As a result, a triple freeze-thaw cycle led to a titer increase of 1 $\log_{10}$; therefore, this step was included in the optimized virus production protocol.

Furthermore, the possibility of concentrating the virus from cell culture supernatant using ultracentrifugation was considered, which has also been described by other groups [205]. This resulted in a high concentration factor, which was achieved using large starting volumes and small re-dispersion volumes. Moreover, the method allowed the re-dispersion of the virus in another buffer, which was better suited for the intended experiments, e.g. for calibration of pH. This sterile filtered colorless aqueous isotonic sodium chloride solution with a phosphate-buffered pH of 7.4 (PBS) turned out to be optimal. Therefore, ultracentrifugation was also included in the HEV production protocol.

HEV stock dispersions generated in this manner possessed virus titers of >4 log_{10} focus forming units (ffu)/ml and were thus suitable for inactivation trials, as a 4 log_{10} reduction has been suggested in European standards for virucidal efficiency [212]. An HEV stock dispersion was characterized in more detail [58]. For this purpose, it was subjected to analytical cesium chloride density gradient ultracentrifugation. As result, two fractions of HEV particle species were obtained. This is in accordance with other studies describing two particle species of HEV - quasi-enveloped and non-enveloped - in cell culture supernatant and cells [49], [57], [60]. The fraction with the lower density (1.19 g/ml) contained about 16 times more genome copies/ml than that with the higher density (1.29 g/ml). By electron microscopy, the lower density fraction showed quasi-enveloped HEV particles, whereas in the higher density fraction non-enveloped HEV particles were found. Therefore, the HEV dispersion represents a mixture of quasi-enveloped and non-enveloped HEV particles, reflecting the situation of HEV particles in blood and in feces, respectively. It is not yet known, which particle types are present in meat and meat products, but a mixture of both particle types is conceivable. Therefore, this situation would also be well simulated with the HEV stock dispersion.

Despite all improvements in the optimized cell culture system, it still remains laboratory intensive and time-consuming. Thus, it allowed only a limited number of biological and technical replicates, which is why a design of two biological replicates with four technical replicates each was chosen for all experiments.

5.3 pH stability of HEV

In this study, the stability of HEV against different pH values was determined. The first series of experiments focused on a wide range of pH values, ranging from pH 1 to pH 10 for 3 hours at RT. Only pH 1 resulted in complete inactivation and pH 10 resulted in strong, but not complete, inactivation. I contrast, in the range of pH 2 to 9, only slight decreases in infectivity were observed, indicating a high pH stability of HEV. Based on published data, a very similar pH stability was found for Tulane virus, a surrogate of human norovirus, which also exhibits high stability (<1 log_{10} infectivity reduction) in the pH range of 2 to 9 [213]. Other related viruses such as feline calicivirus and canine calicivirus are only rapidly inactivated at pH values <3 and >9

[214], [215], whereas the murine norovirus is stable in the pH range of 2 to 10 [214]. Hepatitis A virus (HAV) showed high stability even at pH 1 [216]. It can be speculated that the high stabilities of these enteric viruses help them to remain infectious over the large pH range present in the GI tract, thus enabling infection of the host organism via the intestine. The data generated in this thesis support this hypothesis, as HEV is also orally transmitted and needs to survive the passage of stomach and intestine for successful infection.

The second experimental series focused on pH values typically found during the ripening of meat and the curing of meat products using pH 4.5 to 6.5 with D/L-lactic acid, which is the main pH regulator during meat curing. The selected conditions simulate typical conditions that occur during meat cutting (4 °C) and during the ripening phase of short-ripened raw sausages (23 °C). Compared to the control sample without adding D/L-lactic acid, only slight reductions in infectivity were found by incubation at 4 °C or 23 °C for 7 days, with the highest decrease (about 0.5 $\log_{10}$ ffu/ml) at the higher temperature and lowest pH values. Data on comparable studies with other enteric viruses or their surrogates are very limited. One study dealt with the enteric cytopathic human orphan (ECHO) virus, a human enterovirus. This virus showed no significant decrease in infectivity after a 7-day incubation with pH 4.5 to 6.0 at RT or 4 °C [217]. Feline calicivirus decreased its infectivity by about 2 $\log_{10}$ with pH 4.5 after a 7-day incubation at RT, whereas it remained stable under the same conditions at 4 °C [217]. From our data, it can be concluded that HEV cannot be sufficiently inactivated under pH conditions usually present during production of meat or raw sausages.

5.4 Salt stability of HEV

The stability of HEV against different salt concentrations and salt combinations was determined in two experimental series. First, HEV stability against extreme sodium chloride concentrations up to 20% should be tested in comparison to phosphate-buffered saline (PBS), which contains a low sodium chloride concentration of 0.8%. In addition, either 0.015% sodium nitrite or 0.03% sodium nitrate was added as these salts are commonly used up to these concentrations during curing of meat products. After incubation for 24 hours at 23 °C, none of these salt combinations significantly

reduced HEV infectivity compared to the control sample. Even an extreme sodium chloride concentration of 20% in combination with sodium nitrite or sodium nitrate concentrations, which represent maximal allowable values for meat products by the EU, did not result in any appreciable (<0.5 log_{10} ffu/ml) inactivation. This indicates a very high resistance of HEV against high salt concentrations.

In the second experimental series, the stability of HEV against salts under conditions used in raw sausage production should be determined. To simulate conditions of a short-ripened raw sausage, 2% sodium chloride with or without addition of sodium nitrite was used for incubations for up to 6 days at 22 °C. To simulate conditions of a long-ripened raw sausage, HEV samples were incubated with 2% sodium chloride with or without adding of sodium nitrite or sodium nitrate for up to 8 weeks at 16 °C. Under both conditions, only very low HEV titer reductions (up to 0.5 log_{10} ffu/ml) were found under these conditions compared to samples without adding salts. It can be concluded from these data that salt conditions, which are commonly present in raw sausage production, do not lead to any appreciable HEV inactivation.

Stability of viruses against salts has only scarcely been investigated so far. Published data for the ECHO virus indicate a similar high stability against high sodium chloride concentrations [217]. After a 7-day incubation with 20% sodium chloride at 4 °C or 20 °C, no inactivating effect was observed. Addition of 0.01%, 0.015% or 0.02% sodium nitrite to a 2% sodium chloride solution also did not result in ECHO virus inactivation. In contrast, feline calicivirus shows a sodium chloride-dependent inactivation property, which is dependent on the duration of incubation and temperature [217]. After 3 hours incubation with sodium chloride concentrations up to 20% at 4 °C or 20 °C, no infectivity loss was recorded compared to PBS, but significant infectivity reductions were measured after a 7-day incubation at 20 °C, with greater reductions at higher sodium chloride concentrations. As feline calicivirus is a virus that infects the oral cavity of cats and is mainly transmitted by direct contact, a lesser environmental stability seems to be sufficient for this virus, as compared to enterically transmitted and foodborne viruses such as ECHO virus and HEV.

5.5 High hydrostatic pressure processing stability of HEV

High hydrostatic pressure processing (HPP) is a method for food preservation that has been increasingly used in recent years [218]. In this process, microorganisms are inactivated by short application of very high pressure. Since an inactivating effect of HPP on several viruses has been described recently [218], HEV should be investigated here.

The first experimental series focused on the investigation of HEV stability against linearly increasing pressures from 100 to 600 MPa at two temperatures for a fixed time of 2 minutes. As a result, a quasi-linear decrease in HEV infectivity with increasing pressure was observed, with the strongest inactivating effect at 600 MPa (>3.5 $\log_{10}$ ffu/ml) compared to the control sample at atmospheric pressure. Dependency on the pressure height has also been shown for other viruses, e.g. human norovirus in oysters was not completely inactivated at 400 MPa at 25 °C for 5 minutes, but only at 600 MPa at these conditions [219]. In addition, an effect of temperature was evident in the range of 100 to 500 MPa, where the infectivity was up to 1 $\log_{10}$ ffu/ml more decreased at 4 °C than at 20 °C. Dependency on temperature has also been described for other viruses, e.g. for murine norovirus, which is more efficiently inactivated by HPP at 0 °C, 4 °C or 5 °C as compared to 20 °C [220], [221].

In the second experimental series, the widely used 400 MPa was treated as a constant. Under this defined pressure, a time-course analysis was performed with regard to HEV stability at two different temperatures. As a result, a slight exponential decrease in HEV infectivity over time was observed, with the greatest inactivation after 10 minutes of high pressure treatment (2 - 2.5 $\log_{10}$ ffu/ml) compared to the respective control sample. The results indicate that longer pressure holding times can increase the inactivation of HEV at 400 MPa, but even 10 minutes at this pressure are not sufficient to completely inactivate HEV.

To better understand the mechanism of HEV inactivation by HPP, electron microscopic images were taken of an untreated HEV sample and a sample after exposure to 600 MPa for 2 minutes at 20 °C. In the untreated sample, small particles were identified, which strongly resemble HEV particles from other studies. In contrast, in the high-pressure treated sample, only misshapen structures were found, indicating disassembled and distorted virus particles. Similar effects have been

observed for other viruses, e.g. for murine norovirus or rotavirus [221], [222], indicating that the virus particles are mechanically destroyed by HPP treatment.

Comparability of the data with that of other viruses is difficult, as different matrices and temperature/pressure/time regimes are mostly applied in the published studies. Nevertheless, some comparable data on enteric viruses are available. For example, feline calicivirus shows >7 log_{10} infectivity reduction after 1 minute treatment at 450 MPa and 15 °C in cell culture medium and mineral water [223]. Murine norovirus shows >6 log_{10} infectivity reduction after 5 minutes treatment with 450 MPa at 20 °C in cell culture medium [224]. In contrast, HAV shows only a 2 log_{10} infectivity reduction after 5 minutes treatment with 400 MPa at 20 °C in cell culture medium with increased salinity [225]. Thus, HAV behaves more similarly to HEV under HPP treatment than the other viruses. However, HEV even shows a slightly higher stability compared to HAV under HPP. Thus, compared to other enteric viruses, HEV must be considered relatively stable to HPP treatment.

In summary, HEV can certainly be inactivated by HPP treatment, which should therefore be considered for treatment of meat products. However, the data show that extremely high pressures of 500 MPa and more are required for efficient inactivation (>3 log_{10} ffu/ml). As for a virucidal efficacy - at least in the context of disinfectants - the infectivity should be reduced by more than 4 log_{10} [212], only the highest pressure of 600 MPa can be considered to sufficiently inactivate HEV.

5.6 Drying stability of HEV

One important question for the assessment of HEV transmission pathways is, how stable the virus is after drying. The knowledge about the HEV drying stability might be used to assess the risk of virus transmission via environmental pathways, but will also be useful for development of hygiene measures during food production. Three different series of experiments should therefore assess the stability of HEV against the drying process itself, as well as on the stability during subsequent storage at RT or by cooling.

Only small decreases in infectivity (0.1 to 0.7 log_{10} ffu/ml) were found due to the process of drying, and the decreases were even smaller by addition of bovine serum

albumin (BSA). It can therefore be concluded that HEV is not efficiently inactivated by drying. Other enteric viruses also show high stabilities against the drying process itself. A published study which compared HAV, rotavirus, poliovirus and adenovirus indicated the highest stability against drying for HAV, which showed comparable infectivity decreases as determined here for HEV [226].

According to Sánchez & Bosch [227], the resistance of a virus to drying *per se* plays the decisive factor in its continued resistance on dry surfaces. Since HEV shows on average only a minimal decrease in terms of infectivity in this context, long-lasting persistence in dried condition must be expected. To test this hypothesis, HEV samples already dried on the different materials were stored at 23 °C and 26% relative humidity (RH) for up to 8 weeks, which resemble conditions in rooms, groceries or kitchens. As a result, a continuous decrease of HEV infectivity over time could be observed. The reduction in infectivity ranged from 2.8 to 4.3 $\log_{10}$ ffu/ml after 4 weeks, depending on the material and BSA addition, whereas no residual infectivity could be detected after 8 weeks of incubation. To test conditions resembling that are usually present in refrigerators and cooling facilities, HEV was stored for up to 8 weeks at 3 °C and 98% RH. By this, a much higher stability could be observed over the whole investigated period compared to the ambient conditions. In addition, the inactivation was steeply more depend on the respective surface material. For instance, at 8 weeks, plastics showed only a very low (0.5 - 1 $\log_{10}$ ffu/ml) inactivation effect, followed by ceramics and steel, whereas wood showed a strong inactivation (3.8 $\log_{10}$ ffu/ml). In addition, a slight stabilizing effect of BSA was observed here. A comparison with published results of drying stability testings of other enteric viruses indicates similarities with that assessed here for HEV. For example, HAV, poliovirus and adenovirus also showed higher stabilities at lower temperature (4 °C) compared to higher temperature (20 °C) [226], although the effect is less pronounced than for HEV. Comparing the stabilities on different materials, HAV also exhibited higher stability on smooth surfaces (aluminium, ceramics) compared to porous ones (paper).

In summary, HEV shows a high stability against drying *per se* and subsequent storage under different conditions. Therefore, remaining infectious virus has to be expected for long time on surfaces, which were initially contaminated with HEV. The use of suitable surface materials during food production may also support hygienic

measures, as the material type has been shown to have large effects on HEV stability.

5.7 Summary on the current knowledge on HEV stability and inactivation

The results of the presented studies complement the knowledge on HEV stability and inactivation published in the recent years. Table 3 summarizes these findings, which include thermal treatments, storage in liquid matrices and under dried conditions, treatments with high hydrostatic pressure as well as treatment with UV light. In addition, results of chemical treatments including pH, various salt combinations and concentrations, free chlorine as well as alcoholic disinfectants are shown. In conclusion, HEV turns out to be a virus with a high stability against physical and chemical treatments. It seems to be highly resistant against salt and most alcoholic disinfectants. In the other cases, only the stronger conditions (e.g. high temperature, high hydrostatic pressure, high UV doses, extreme pH or high chlorine concentrations) can inactivate this virus efficiently.

However, the total number of studies conducted is still low. Except for higher temperature and high pressure treatment, all other influencing factors have been investigated in only once. Regarding the applied detection methods, only the studies on heating of liver pâté and liver were investigated in the pig model, whereas all other studies used cell culture for infectivity measurement. However, the applied cell culture methods also show differences. For example, Imagawa [228] and Nasheri [229] determined HEV infectivity by measuring generated virus genomes using reverse transcriptase quantitative PCR (RT-q-PCR) from cell culture supernatants, whereas all other authors used immunofluorescence to show virus proteins in infected cells. As the remnants of the virus genome from the inoculated virus itself can be detected for a longer time in cell culture supernatants, the RT-q-PCR-technique might overestimate the amount of infectious virus compared to the immunofluorescence technique. Thus, comparability among the studies is generally difficult, because heterogeneous study designs are prevailing.

Parameter	Matrix	Result	Method	Reference
Heat	Liver pâté	Complete inactivation after 20 min at 71°C	Inoculation in pigs	[199]
	Liver	Complete inactivation after 5 min at 71°C	Inoculation in pigs	[198]
	Minced meat	Complete inactivation (>3 log_{10}) after 5 min at 70°C	Cell culture	[228]
	Cell culture medium	Complete inactivation (>4 log_{10}) after 2 min at 70°C	Cell culture	[204]
Storage (liquid)	Cell culture medium	Complete inactivation (>4 log_{10}) after 8 weeks at RT; 3 log_{10} reduction after 8 weeks at 4°C	Cell culture	[204]
Storage (dry)	PBS/BSA on surfaces	Complete inactivation (>4 log_{10}) after 8 weeks at RT (all surfaces); after 8 weeks at 3°C reduction of <1 log_{10} on plastics, 2 log_{10} on steel and ceramics, >4 log_{10} on wood	Cell culture	[197] (this thesis)
High hydrostatic pressure processing	Liver pâté	0.5 log_{10} reduction after 5 min with 600 MPa at RT	Cell culture	[229]
	PBS	2 log_{10} reduction after 2 min with 400 MPa at 4°C (1 log_{10} at RT); 4 log_{10} reduction after 2 min with 600 MPa at 4°C and RT	Cell culture	[230] (this thesis)
UV light	Water	4 log_{10} reduction with 231.94 J/m^2	Cell culture	[206]
pH	PBS	Complete inactivation (>3 log_{10}) with pH 1 and 10 after 3 h at RT; scarcely any inactivation with pH 2 - 9	Cell culture	[58] (this thesis)
Salt	PBS	Scarcely any inactivation with 20% NaCl after 24 h at 23°C; compared to PBS no inactivation with 2% NaCl, 2% NaCl + 0.015% $NaNO_2$ or + 0.03% $NaNO_3$ after 6 d at 22°C or after 8 weeks at 16°C	Cell culture	[231] (this thesis)
Chlorine	Water	2 log_{10} reduction after 1 min with 5 mg/l free chlorine	Cell culture	[205]
Alcohol-based disinfectants	Cell culture medium with BSA	>4 log_{10} reduction with one product; <1 log_{10} reduction with 4 other products	Cell culture	[232]

Table 3. Summary of current studies on HEV stability and inactivation due to different physical and chemical parameters.

5.8 Importance of the results for food production and food safety

When discussing the results in the context of food safety, raw meat (including liver) and raw meat products from infected pigs can be considered as the most important sources of human infection with HEV in Germany. For instance, Faber [233] conducted a case-control study, in which “pork meat (e.g. grilled)”, “wild boar”, “pork liver” and “liver sausage or liver pâté” showed the highest odds ratios indicating a risk of getting diseased with hepatitis E by consumption of these food types. From the perspective of food safety, the production process of food may explain these risk products and also imply possibilities to produce food that is more safe regarding contamination with HEV. As shown in Figure 15, the pig is the source for various meat products, which are each pass through a specific production process, which might inactivate HEV or not and therefore represent a risk for HEV infection or not.

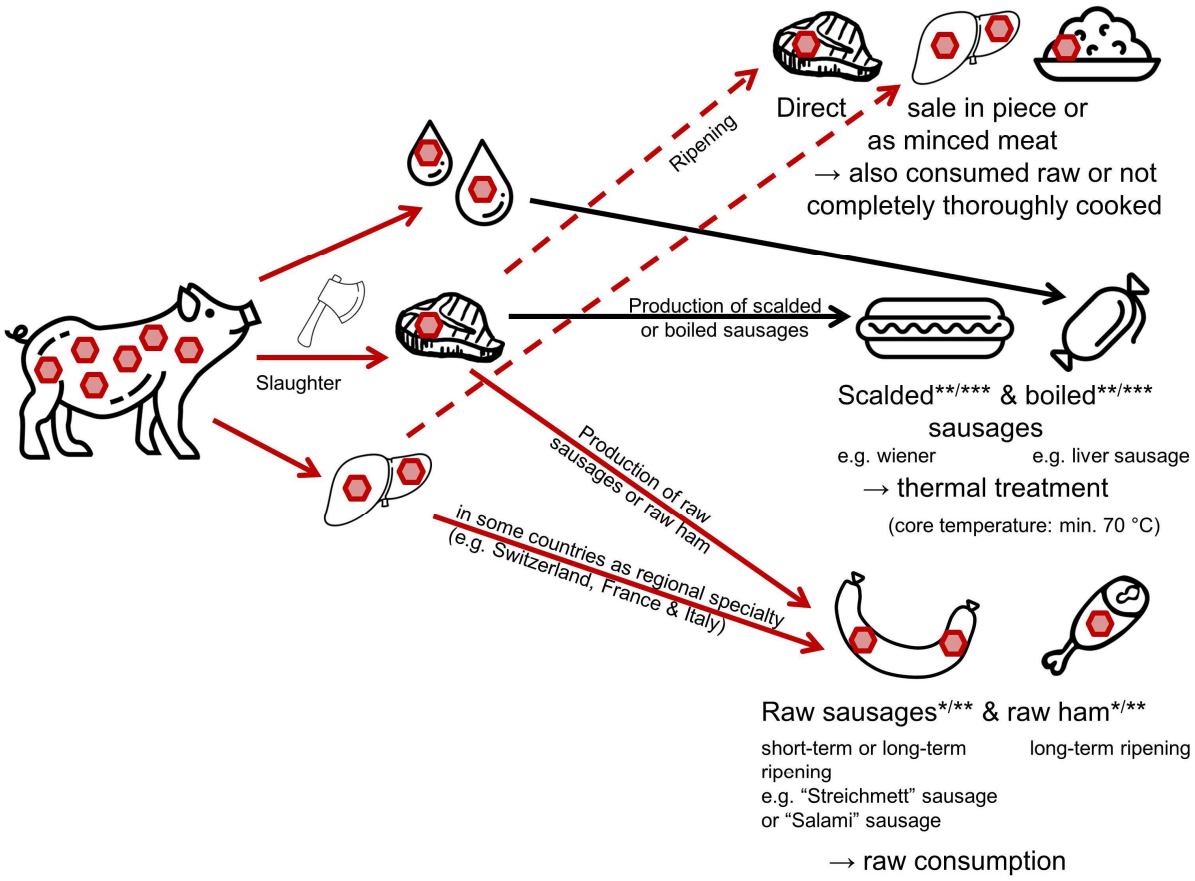

Figure 15. The pig as food supplier. This figure shows the use of body components from the domestic pig in the course of food production. The continuous red arrows indicate a probable high load of infectious HEV in the final product, whereas the dashed red arrows indicate a possible load of infectious HEV in the final product, depending on the preparation by the end consumer. For the black

arrows, infectious HEV in the final product cannot be assumed, due to thermal treatment. * = including curing, ** = partially smoked, *** = partially cured.

To assess the potential for infection, it is helpful to classify the various meat products into commodity groups with similar production processes. The first major commodity group is **meat**, which is offered for sale in a piece or as **minced meat**. It generally undergoes a ripening process of a few days beforehand, depending on the type of meat. In the case of pork, this process takes 3 days, whereas for beef it can last up to 14 days [234], resulting in tenderization and umami meat flavor [234]. From slaughter to sale, the meat is stored at around 2 to 4 °C and 85% RH. For HEV genotype 3, storage in liquid (cell culture medium) at 4 °C led to an infectivity decrease of around 1.5 $\log_{10}$ ffu/ml after 14 days [204]. Thus, under these conditions, infectious HEV particles that may be present in the meat at slaughter will not be efficiently inactivated at this temperature and time-period. In addition to temperature, pH is changing during ripening [234]. Before slaughter, the pH is around 7.3, which drops to around 6.0 at 1 hour after slaughter. Thereafter, the pH drops to the minimum of around 5.3 after 24 hours, before it increases again at ripening to a slightly acidic pH range. Thus, meat is exposed to pH values down to 5.3 within 14 days. According to the results from the study presented here, no inactivation of HEV can be expected under these conditions. Taken together, infectious HEV has to be expected in this commodity group at the time of sale, if it was present in the meat at slaughter.

The second major commodity group includes **scalded and boiled sausages**. Both types of sausage are also subjected to thermal treatment in the course of their production [234]. For this heating process, a core temperature of at least 70 °C has become established among the manufacturing companies, which ensures sufficient stability with regard to spoilage by microorganisms [234]. In practice, core temperatures of around 72 °C are frequently used [234]. However, only reaching the core temperature is controlled, whereas the holding time is not clearly defined [234]. At 70 °C, HEV-3 infectivity in cell culture medium was reduced for 3.0 $\log_{10}$ ffu/ml after 1 minute and >4.0 $\log_{10}$ ffu/ml (complete inactivation) after 2 minutes [204]. Other studies also achieved complete HEV inactivation under similar temperatures, but with slightly different incubation times [198], [199], [228]. For instance, in the case of liver pâté at 71 °C, complete HEV inactivation could only be achieved after 20

minutes [199]. In summary, only a very low, if any, infectious HEV load can be expected in boiled and scalded sausages after applying sufficient heating holding times (2 - 20 minutes) during their production process.

The third major commodity group includes **raw sausages**. In this commodity group, the finished products must be differentiated according to their specific production processes. A differentiation can be made into slowly ripened and fast ripened raw sausages. The slow ripening process also includes whole ripened raw meat cuts, such as raw ham. Figure 16 shows these raw sausage production processes.

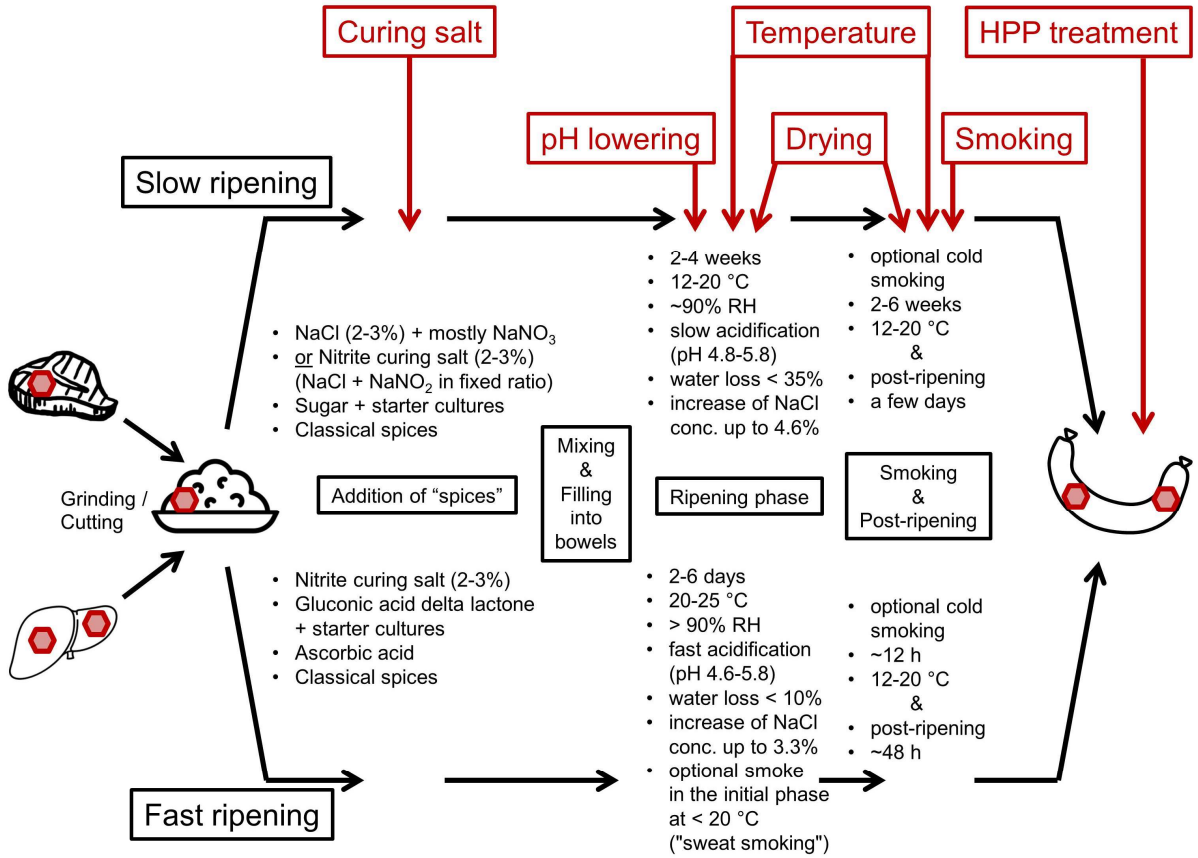

Figure 16. The process of raw sausage production. Above the production by slow ripening, below the production by fast ripening. The red arrows represent different parameters, which could possibly have an influence on HEV infectivity during both manufacturing processes.

As evident from the Figure, many different parameters can influence HEV infectivity during production. The first is the addition of curing salt. It consists of sodium chloride and a curing agent such as sodium nitrite or sodium nitrate. It is shown in the

presented study, that neither usual sodium chloride concentrations nor maximal permissible concentrations of the curing salts can inactivate HEV under conditions prevailing during slow or fast raw sausage ripening [231]. In addition to salt, the pH, which can reach 4.6 in case of fast-ripened raw sausages, should be considered. In the presented study, only a reduction in HEV infectivity of around 0.5 $\log_{10}$ ffu/ml was reached at this pH after 7 days at 23 °C compared to the control sample [58]. However, the infectivity loss compared to the starting sample was already around 1.3 $\log_{10}$ ffu/ml, indicating that the storage time seems to play a major role in this process. The third variable is the storage temperature. In cell culture medium, HEV infectivity reduction was about 1.7 $\log_{10}$ ffu/ml after 7 days storage in cell culture medium at RT whereas after 8 weeks, no residual infectivity (titer reduction >3.8 $\log_{10}$ ffu/ml) could be detected [204]. In contrast, infectious HEV could be detected after incubation of HEV in PBS for 8 weeks at 16 °C, with a titer decrease of 2.2 $\log_{10}$ ffu/ml [231]. However, it has to be considered that during the ripening phase and thereafter, a loss of water due to drying of the raw sausage happens; therefore, comparability with cell culture medium or PBS is questionable. Due to the water loss, the activity of water (a_w) value in the raw sausage decreases, the salt concentration increases and with it also the osmotic pressure. However, our study showed that HEV is also stable against extremely high salt concentrations of 20% sodium chloride [231].

In summary, by looking at the distinct processes occurring during raw sausage production, which include increase of salt concentration, lowering pH and storage at certain temperatures for longer time intervals, none of these processes can efficiently reduce the amount of infectious HEV. Therefore, the presence of infectious HEV can be expected in raw sausages, if it was produced from contaminated meat. However, it has to be considered that all of these studies investigated only each of the treatments as single factors, and a combination of them might lead to a more pronounced HEV inactivation. In addition, these studies have been done only with HEV in liquid solutions, and studies directly in the food are desirable in the future.

Additional treatments can be performed in special cases of raw sausage production. This may include a cold smoking process, which follows the ripening phase. By this, the aroma and color changes, and preservation by aromatic hydrocarbons and further lowering the a_w value can be achieved [234]. Smoking may

therefore also affect infectivity of pathogens. However, its effect on HEV infectivity has not yet been investigated and should be a task for future investigations. In addition to smoking, HPP treatment after finishing the products could be optionally performed. This purely physical process is of no concern to the consumer and has been used successfully for a while in several countries, e.g. in Spain for the preservation of sliced cooked ham [218]. Although several studies have shown an inactivating effect of HPP on viruses [218], HEV showed a considerably higher stability compared to other viruses in our study [230]. However, by applying the highest tested pressure of 600 MPa for 2 minutes, HEV infectivity could be reduced for about 4.0 $\log_{10}$ ffu/ml, indicating that these conditions are sufficient for production of safe food. However, further evaluations of the HPP stability of HEV in the distinct sausage matrix are needed in order to finally assess the efficiency of this treatment.

5.9 Importance of the results for environmental contamination

Possible transmission pathways of HEV through environmental contaminations are manifold and complex (Fig. 17). The probability of a specific transmission route depends crucially on the stability of the virus against specific environmental factors. Especially, the influence of stability during and after drying is important and will be mainly considered in this Chapter.

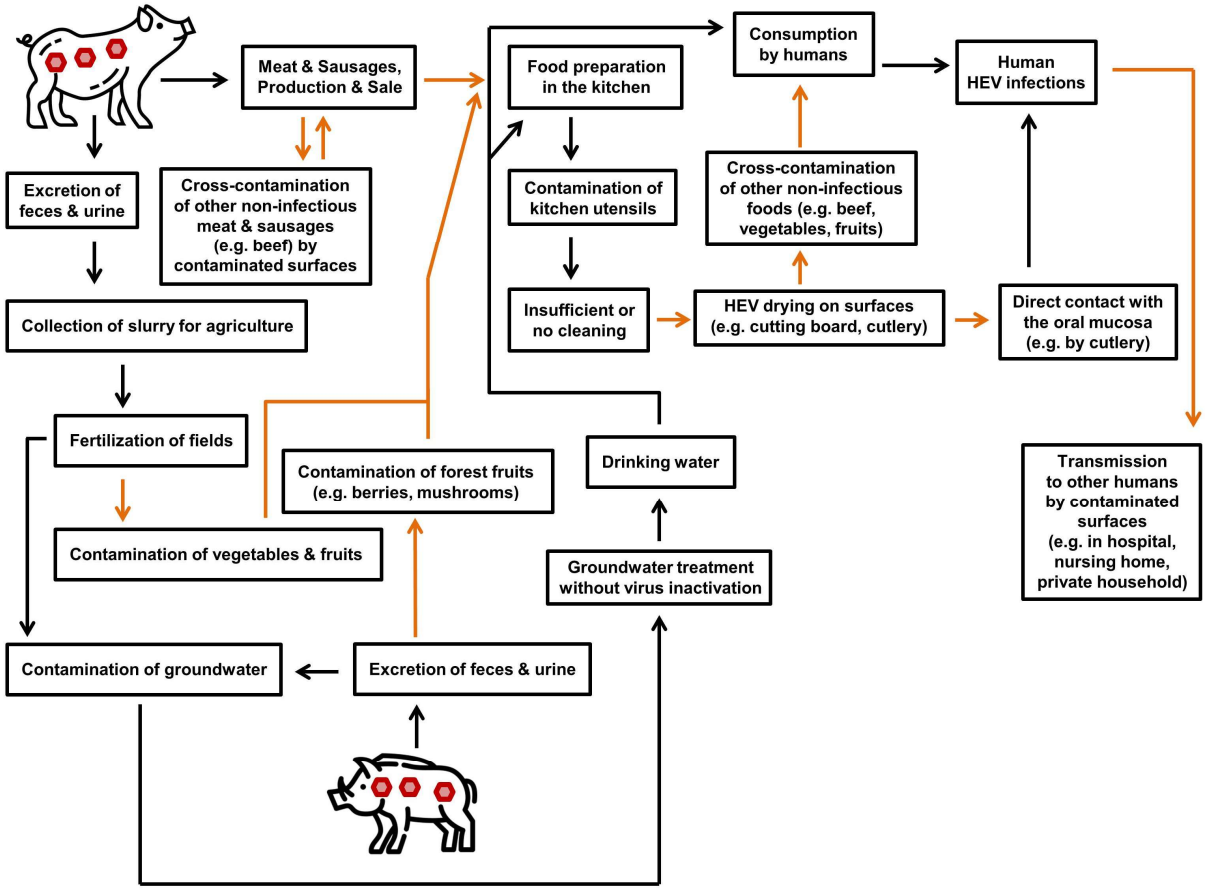

Figure 17. Environmental transmission of HEV-3. Detailed illustration of possible transmission routes of HEV genotype 3 due to environmental contamination originating from the reservoir animals domestic pig and wild boar to humans. For the brown arrows, drying or dry storage on surfaces can play an important role.

As illustrated in Figure 17, the importance of drying for the transmission routes can be grouped in four different areas. First, transmission by **contaminated surfaces during meat and sausage production** may be considered. For technological and hygienic reasons, the temperature in slaughterhouses, processing facilities and in sales counters is usually low, with high humidity. The presented study showed that dried HEV exhibits extraordinary stability at 3 °C and 98% RH for at least 8 weeks, especially on smooth surfaces [197]. Thus, a very long persistence of dried HEV originating from contaminated meat or pig excretions has to be expected on commonly used surfaces in food production (e.g. plastics and steel). A strict hygiene concept with regard to the cleaning of utensils such as knives or cutting boards,

surface disinfection as well as personal hand hygiene is therefore mandatory in order to avoid cross-contaminations between different types of meat and their products.

The second area concerns the **contamination of non-animal food by excreta of pigs and wild boars**. Whereas the excrements from indoor housing are collected and brought to the fields as slurry for fertilization, those of wild animals are distributed in the forest and thus also reach mushrooms and berries. HEV can be excreted in high concentrations from infected animals via the feces [72]. In addition, infectious virus has also been detected in urine from an HEV-infected monkey, showing the potential risk that also emanates from this matrix [69]. If these excreta come in contact with field or forest fruits, contamination with HEV can therefore occur. This is confirmed by studies showing the presence of HEV RNA on plant-based food such as berries [235]. The distinct stability of HEV on living biological surfaces such as berries has not yet been investigated. However, the data of the presented study on stability of dried HEV at RT on inanimate surfaces indicates, that it will be stay infectious for several (up to four) weeks [197]. The influence of UV light on HEV must of course be considered, which might shorten the survival time of infectious HEV under outdoor conditions [206]. A possible preventive measure in this context would be HEV testing of the slurry before it is brought onto the fields. Heat treatment of slurry before use may also be considered.

The third area concerns the **contaminations in domestic and public kitchens** including canteens, school kitchens and kitchens in old people's and nursing homes. Contaminated food acquired through the areas 1 and 2 may contaminate surfaces during food preparation in these kitchens. Our study showed that, depending on the material and conditions (temperature, RH), dried HEV particles can remain infectious on surfaces at RT for several weeks [197]. If kitchen utensils are not cleaned correctly, cross-contaminations with other foodstuffs may occur, e.g. by contaminated cutting boards or insufficiently cleaned cutlery. In addition, insufficiently cleaned cutlery may pose a risk for smear infections. A particular risk may arise from storage in the refrigerator, e.g. by using storage containers as source of cross-contaminations, because a particularly long HEV persistence is to be expected under these cool and humid conditions [197]. As a conclusion, proper hygiene rules, e.g. using water with high temperatures and sufficient detergent for dishwashing, regular

hand washing and separating pig meat preparations from preparations of food intended for raw consumption, can avoid HEV transmissions in this context.

The fourth area concerns **surface contaminations by infected persons**. These may pose a high risk, especially in a setting with other patients belonging to a risk group (e.g. immunosuppressed people). For example, dried blood, tissue fluids, feces and urine in hospitals or nursing homes can contaminate surfaces, which are expected to be infectious for several weeks based on our study [197]. Also in this area, the implementation of a well-designed hygiene concept is necessary. This is complicated by the fact, that currently used disinfectants are mostly not tested for their inactivating activity on HEV. Only one study investigating alcohol-based disinfectants has been published so far; however most of these showed only a very low effect on HEV infectivity [232]. Therefore, it is an urgent need on disinfectant testing, in order to provide hospitals with tools for efficient decontamination of surfaces from HEV contaminations.

5.10 Importance of the results for medical products

A further important risk for HEV infection is receiving blood and blood products, e.g. in the context of transfusions. During production of blood products, pathogens should be inactivated by application of specific treatments. In Europe, there are three different pathogen inactivation systems with a CE label available, which are therefore also marketable within the EU [159]. In Germany, an additional approval is required, which is currently held by only one of the three systems [159]. All three systems are based on the inactivation of nucleic acids and are therefore in principle suitable for all blood products that do not contain nucleated cells as an active ingredient, which include erythrocyte concentrates, platelet concentrates and therapeutic plasma [159].

The INTERCEPT^{TM} system, approved in Germany for pathogen inactivation in platelet concentrates, generally has a wide inactivation spectrum [159]. It is based on an interaction of the specific substance amotosalen with nucleic acids, which leads in the presence of UVA light to crosslinking and inactivation of pathogens [159]. However, efficiency of inactivation is dependent - among others - on the accessibility of the nucleic acid, which may be shielded by a tight virus capsid. Indeed, the INTERCEPT^{TM} system shows high efficacy against enveloped viruses, whereas

efficacy against non-enveloped viruses is limited or even non-existent [159]. It also appears to be ineffective against HEV, as transmission of HEV to two patients by transfusion of plasma treated with this system has been reported [236].

An additional effect noted in platelet concentrates treated with this system is a pH decrease due to lactic acid formation as a result of anaerobic energy production due to platelet activation [159]. Lower pH values may also lead to further pathogen inactivation. However, in some studies, no significant pH differences were found during 7 days of storage between samples treated with this system and the respective untreated controls [237]–[239]. The optimal pH range for storage of platelet concentrates for up to a maximum of 5 days is between 6.5 and 7.0 at a temperature of 20 - 24 °C under constant agitation [240], [241]. Below pH 6.2 or above 7.9, damage to platelet function occurs [240], [242]. The data of our study show that under these conditions, HEV inactivation by pH does not occur [58]. Thus, it can be concluded that treatment of platelet concentrates with INTERCEPT^{TM} does not result in a pH decrease, that could inactivate HEV under these conditions. Since similar pH decreases have been described for the other two systems that are marketable in the EU [159], no HEV pH inactivation can be assumed here either. In addition, the results of our studies on HEV resistance against salt, drying and high pressure may also be useful for further developments of pathogen inactivation systems for medical products in the future.

5.11 Conclusions

Hepatitis E has been identified as an increasing concern in many European countries including Germany during the last years. Therefore, the elucidation of the transmission pathways of HEV and the development of counteracting measures are of high priority. For both fields of research, the knowledge on the stability of HEV against physical and chemical factors is crucial. However, data on HEV stability and inactivation were only scarcely available at the beginning of this thesis. Therefore, basic data on HEV stability regarding pH, salt concentration, drying and high pressure have been generated here. The data can be used for assessment of HEV stability and inactivation in the fields of food hygiene, environmental science as well as hospital hygiene and safety of medical products.

As a conclusion from the studies, HEV has to be considered as a very stable virus showing high resistance against a broad range of pH values, high salt concentrations, the drying and dry storage process as well as medium pressure. Compared to other viruses, HEV mainly behaves like the most stable enterically transmitted viruses investigated so far, e.g. HAV. Therefore, infectious HEV has to be expected for a long time after contamination of food or surfaces. Only the strongest investigated conditions of extreme pH (pH 1 and pH 10), high hydrostatic pressure (600 MPa for 2 minutes) or application of high temperatures (>70 °C for 2 minutes) have been shown to inactivate HEV efficiently.

These conclusions have consequences for the risk assessment of food production. First of all, the results indicate that HEV infectivity will not be significantly reduced during the production of meat including liver and minced meat. Therefore, the main recommendation, to generally properly cook these food types before consumption [243], is clearly supported by our results. Also, the data indicate that the reduction in HEV infectivity is not complete after passing through the production process of fast or slow ripened raw sausages. As therefore low amounts of infectious HEV may be present in this food types, the recommendation for avoiding consumption of raw sausages especially for risk groups [243], should be maintained. Other meat products, e.g. scalded and boiled sausages, should be considered as safe, if the applied heating regime during production has been performed carefully. Application of HPP to meat products can further increase the safety of food products.

Regarding other transmission routes of HEV, it should be considered that environmental contamination can pose a significant risk. The generated data on the drying and dry storage stability of HEV point to a very high persistence of the virus on various surfaces under different conditions. This indicates that cross-contamination during food production or contamination of non-animal food by porcine excretions might contribute to HEV transmission, which should be counteracted by strict hygienic measures. This is also especially important for the environment of hepatitis E patients in hospital settings, because excretions of them including feces, urine, blood and tissue fluids can contain high HEV amounts and pose a high risk for other hospitalized patients, also in dried condition.

HEV can also represent a risk for the area of transfusion medicine, including blood and blood products. This iatrogenic transmission route should not be underestimated,

as HEV RNA was found in one of 679 to one of 4,252 blood donations in Germany [159]. To counteract this risk, blood and its products for therapeutic use must be routinely tested for HEV in Germany since the beginning of 2020 [244]. However, mass screening of blood donations cannot completely rule out the possibility of low HEV contaminations in the products; therefore, the development and application of safe pathogen inactivation procedures for blood products is still an important issue. The only pathogen inactivation procedure currently approved in Germany is ineffective against HEV, which is in accordance with our results on the absence of HEV inactivation at the associated pH values.

5.12 Future perspectives

The presented studies have some limitations and several research questions result from the generated data, which should be investigated in future.

Generally, only one HEV strain - strain 47832c of genotype 3c - was investigated here. Since there may be differences in stability of different HEV strains, further studies with other HEV types would be desirable. Isolation of different HEV subtypes in cell culture has been described recently [167]; however, it is not known so far if their replication efficiency is sufficient for the desired experiments.

Only a mixture of quasi-enveloped and non-enveloped HEV particles was used in the presented studies. It can be expected that differences in the stability of these two particle types exist, which may have consequences, e.g. for stability of fecally excreted virus (non-enveloped) vs. virus from blood (quasi-enveloped). Therefore, studies with the respective isolated particle types would be useful. Recent studies on inactivation of HEV by alcoholic disinfectants have already compared both particle types, showing slight differences in inactivation between them [232]. A broader investigation of differences in stability of the HEV particle types would therefore be desirable, once the methods for more efficient production and separation of them are available.

One general limitation of the presented studies is, that only PBS with or without adding of BSA was used as matrix. These laboratory conditions may only insufficiently simulate the complexity of matrices such as meat and sausages, blood,

meat juice, non-animal food, animal and human excretions, river water or soil. Therefore, further studies on HEV inactivation directly in those matrices are needed to validate the data obtained so far. However, one pre-requisite would be the development of efficient methods for the investigation of those complex matrices, which would require further optimization of sample preparation procedures and the used cell culture systems.

The generated stability data should be used for generation of mathematical models, which could predict HEV inactivation over a broad range and combinations of parameters. Until now, mathematical models could only be created for HPP [230] and thermal treatment of HEV [204]. The development of broader mathematical models considering diverse parameters is essential for the simulation of more complex scenarios, such as HEV infectivity decrease in animal foods or the environment.

Additional parameters possibly influencing the stability of HEV should also be considered in future investigations. For example, the effect of smoking may be of particular interest, as this technique is widely used during meat processing. Also, the effect of novel food processing technologies such as cold plasma, irradiation or pulsed electric field [188], [245] should be investigated to assess their potential for HEV inactivation.

As mentioned above, stability data on HEV are also important for the development of new systems for pathogen inactivation in medical products, which are derived from human blood or porcine tissue. In this regard, it will be necessary to investigate the specific treatments used during their application, in order to assess their effects on HEV inactivation. On the basis of the results, an optimization of the production processes would be possible, in order to remove residual infectious HEV.

The results of the investigations should be used to establish optimized procedures in production of food and medical products to increase their safety regarding the absence of infectious HEV. The hygienic concepts in food production, wastewater treatment and agriculture should also be harmonized with the findings on HEV stability in the environment, in order to minimize the risk of HEV transmission via these pathways. Last but not least, risk communication on HEV transmission by food, which should include updated consumption recommendations for distinct risk groups, may represent a helpful tool for prevention of hepatitis E cases in humans.

6 References

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7 Appendix

7.1 List of figures

7.2 List of tables

7.3 List of abbreviations

ALT	alanine aminotransferase
a_w	activity of water
BfR	Federal Institute for Risk Assessment (Germany)
BLSV	big liver and spleen disease virus
BLV	Federal Food Safety and Veterinary Office (Switzerland)
BSA	bovine serum albumin
ECHO	enteric cytopathic human orphan
ER	endoplasmic reticulum
FBS	fetal bovine serum
ffu	focus forming units
FU	Free University of Berlin (Germany)
GI	gastrointestinal

HAV	hepatitis A virus
HEV	hepatitis E virus
HIV	human immunodeficiency virus
HPP	high hydrostatic pressure processing
HS	hepatitis splenomegaly
ICTV	International Committee on Taxonomy of Viruses
IEM	immune electron microscopy
IgG	immunoglobulin G
IgM	immunoglobulin M
mRNA	messenger-RNA
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RH	relative humidity
RKI	Robert Koch Institute (Germany)
RNA	ribonucleic acid
RT	room temperature
RT-q-PCR	reverse transcriptase quantitative PCR
SOT	solid organ transplantation
UV	ultraviolet
VLP	virus-like particle

7.4 Supplementary material of own publications

7.4.1 Publication 3

Supplementary Material

Johne *et al.*: Stability of hepatitis E virus at high hydrostatic pressure processing

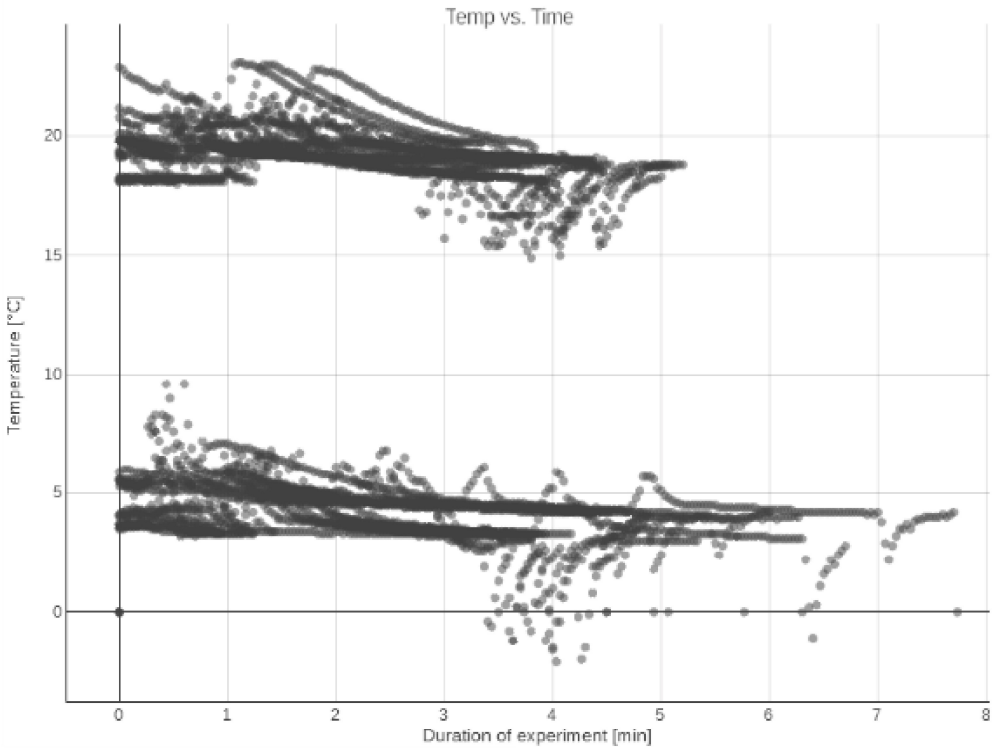


Figure S1. Temperature profiles measured during high hydrostatic pressure treatment of HEV using a thermocouple inside the treatment unit directly above the sample tube. The determined temperatures of all experiments with target temperatures of 20 °C and 4 °C with pressure holding times of 2 min (at 100 MPa to 600 MPa) are shown. The different lengths of the whole experiments are caused by differences in pre-incubation times for temperature adjustment before starting pressure treatment.

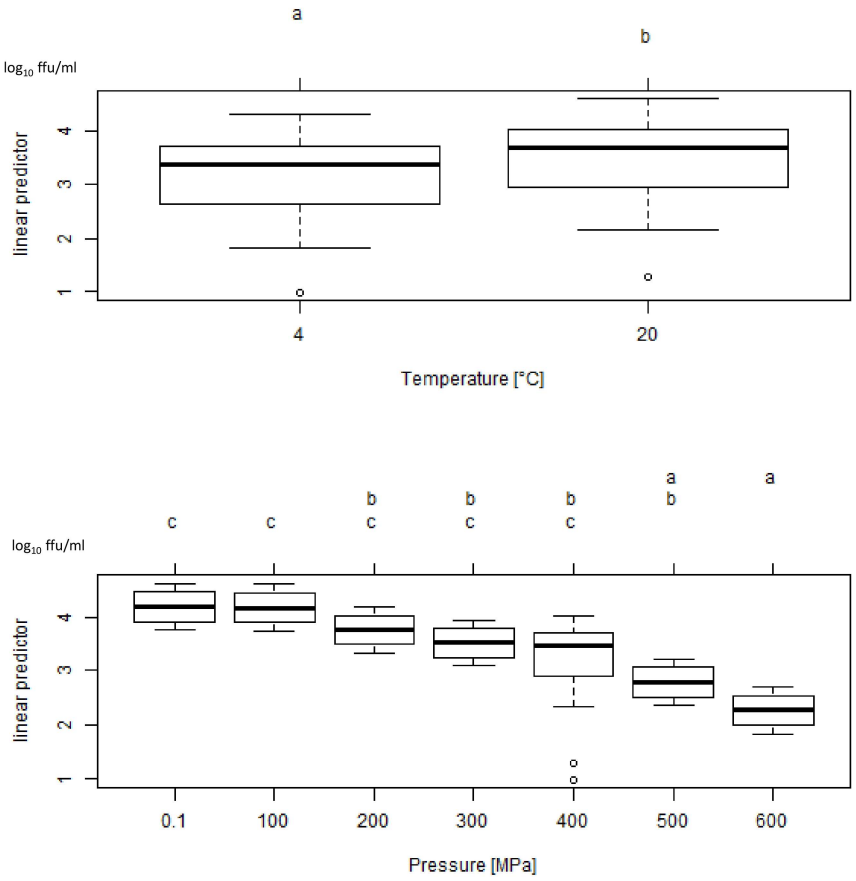
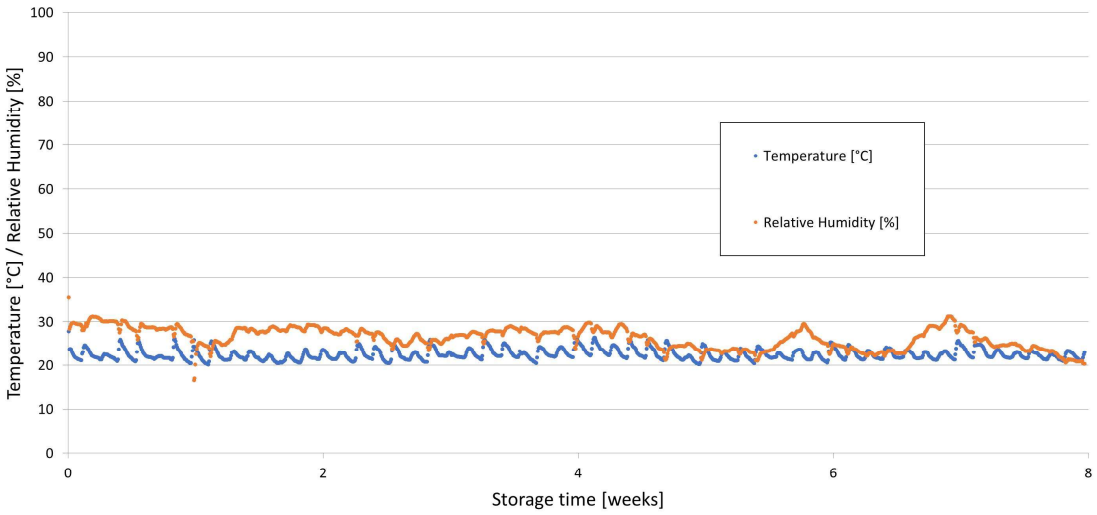


Figure S2. Result of post-hoc pairwise comparison of effects from factor "Temperature" (3A) and "Pressure" (3B) on HEV inactivation based on a linear ANCOVA model. Factor levels not carrying the same letters are significantly different at a 5% family-wise alpha level according to the linear statistical model.

7.4.2 Publication 4

Supplementary Data 1

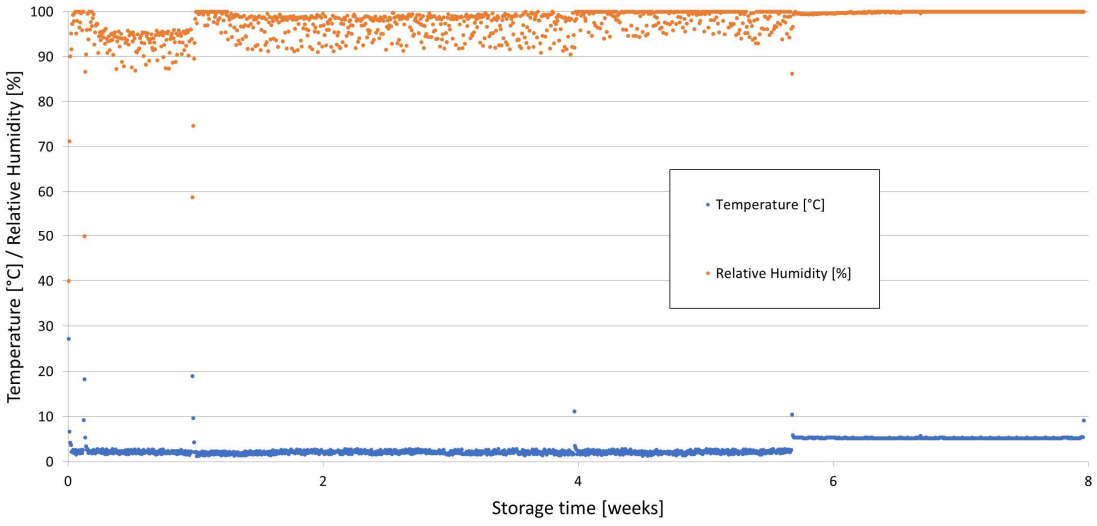
Wolff *et al.*: Stability of hepatitis E virus after drying on different surfaces



Suppl. Data S1. Temperature and relative humidity profiles during storage of dried HEV samples at 23 ° C as recorded by a data logger. The measuring interval was 1 hour.

Supplementary Data 2

Wolff *et al.*: Stability of hepatitis E virus after drying on different surfaces



Suppl. Data S2. Temperature and relative humidity profiles during storage of dried HEV samples at 3 ° C as recorded by a data logger. The measuring interval was 1 hour.

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