

**Discovery of Novel Strains of Animal Hepatitis E Viruses in the United States:
Antigenic and Genetic Characterization, Cross-Species Infection, and Public
Health Implications**

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in
partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Biomedical and Veterinary Sciences

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March 17, 2015

Blacksburg, VA

Keywords: Hepatitis E Virus, cross-species, transmission, zoonosis

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ABSTRACT

Hepatitis E virus (HEV) is an important human pathogen, with pigs and likely other animal species serving as natural reservoirs. There are currently four recognized HEV genotypes that infect humans within the genus *Hepevirus* of the family *Hepeviridae*. Genotypes 1 and 2 are human viruses that are associated with waterborne and fecal-oral transmission in developing countries, while genotypes 3 and 4 have been identified in humans and other animal species and are zoonotic and endemic in both industrialized and developing countries.

In my dissertation research, we identified the first strain of HEV from rabbits in the United States. We subsequently determined the complete genome sequence of the virus. Phylogenetic analyses of the full-length sequence indicated that U.S. rabbit HEV is a distant member of the zoonotic genotype 3, thus raising a potential concern for zoonotic infection. In order to investigate the cross-species potential of rabbit HEV, we then determined its antigenic cross-reactivity with other animal strains of HEV. Additionally, we demonstrated that the novel rabbit HEV could cross species barriers and infect pigs under experimental conditions.

Finally, we attempted to determine the risk factors and sources of foodborne HEV infection in the United States. We detected HEV for the first time from non-liver pork commercial products in the United States and demonstrated consumption of undercooked meat a risk factor for HEV infection. HEV sequences of genotype 3 origin were detected from pork

products purchased from grocery stores in Southwest Virginia. Approximately 6.3% (21/335) of university students tested seropositive for HEV antibodies and, importantly, those with a history of consuming undercooked meats were 13 times more likely to be seropositive. These results further underscore the importance of cooking pork thoroughly and using proper hygiene when preparing meals.

DEDICATION

This dissertation is dedicated to my parents, Tina and Joseph Cossaboom, and sister, Rebecca Cossaboom, for their unwavering love and support.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude and appreciation to my advisor, Dr. Xiang-Jin Meng, for giving me the opportunity to work in his laboratory. There has not been a day during my time in graduate school that I didn't reflect on how lucky I was to be a member of the Meng Lab. The past five years have been a truly wonderful experience and I will forever be grateful for his mentorship, support, and encouragement.

I also owe sincere thanks to the faculty members that served on my doctoral advisory committee, Dr. François Elvinger, Dr. Tanya LeRoith, Dr. Liwu Li, and Dr. Virginia Beuchner-Maxwell, for all of their time, expertise, and support throughout my time as a graduate student.

I would also like to recognize the following former and current members of the Meng Lab: Dr. Nathan Beach, Nicholas Catanzaro, Dr. Dianjun Cao, Qian Cao, Dr. Laura Córdoba Garcia, Barbara Dryman, Dr. Alicia Feagins, Dr. Mary Etna Haac, Lynn Heffron, Dr. Yaowei Huang, Dr. Scott Kenney, Dr. Yanyan Ni, Dr. Christopher Overend, Dr. Pablo Piñeyro, Dr. Sumanth Pudupakam, Dr. Adam Rogers, Dr. Brent Sanford, Sara Smith, Dr. Sakthivel Subramaniam, Dr. Debin Tian, Dr. Heng Wang, Jacquelyn Wentworth, Dr. Danielle Yugo, and Dr. Lei Zhou. Thank you all for all of the help, advice, and friendship you have given me over the past five years. I cannot imagine a better group of people to have spent this time with.

Finally, I would like to thank my parents, Tina and Joseph Cossaboom, sister, Rebecca Cossaboom, grandmother, Joan Sterling, Peggy, and all of my wonderful friends and family members who have stood by me and cheered me on through all of my time at Virginia Tech. I could not have done this without you and love you all.

This research was supported by grants from the National Institutes of Health (AI050611 and AI065546).

ATTRIBUTIONS

All of this work would have been impossible without the dedication and cooperation of many colleagues that contributed to the research, writing, and editing of all of the projects in this dissertation.

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GENERAL INTRODUCTION

Hepatitis E virus (HEV) causes an enterically transmitted viral disease, and is now recognized as an important human pathogen. HEV belongs to the family *Hepeviridae*, and at least four recognized genotypes of HEV are known to infect humans (4). Genotypes 1 and 2 strains of HEV are restricted to the human host and are typically associated with fecal-oral and waterborne transmission in developing countries with poor sanitation infrastructure. Genotypes 3 and 4 have an expanded host range, including humans, pigs, and several other animal species, and are known to be zoonotic and cause endemic and sporadic infection mostly in industrialized countries (4, 7, 8, 14).

Human infection by the small, non-enveloped single-stranded RNA virus typically runs an asymptomatic course, but infection can present with symptoms typical of acute viral hepatitis (4). Mortality rates are generally lower than 1%, however, the case-fatality risk in pregnant women has been known to at least 20% (13). There is also increasing documentation of hepatitis E disease progression into chronicity in immunocompromised patients, such as organ-transplant recipients or patients with concurrent HIV or other viral hepatitis infections (3, 6, 15). These cases of chronic hepatitis E in immunocompromised individuals are almost exclusively caused by the zoonotic genotype 3 HEV of swine origin, thus raising serious food safety concerns over HEV transmission (15).

HEV is unique among the other hepatitis viruses in that it has known animal reservoirs, the first of which was discovered in 1997 to be domestic pigs in the United States (9). Since this initial discovery, pigs have been recognized as animal reservoirs for HEV transmission to humans, and the consumption of undercooked pig livers has been increasingly linked to cases of hepatitis E throughout the world (1, 2, 10-12, 14, 16, 17). Because a significant proportion of the

individuals in the United States and other industrialized countries do not consume pig liver, the relatively high seroprevalence of anti-HEV antibodies in these countries suggests other potential source(s) of infection. Unfortunately, there has been no investigation into the presence of HEV in non-liver commercial pork products in the United States

A novel strain of HEV in rabbits was identified from farmed rabbits in China in 2009 (5, 18). The presence or prevalence of HEV in rabbits in the United States was unknown prior to my dissertation research. Investigation of rabbit HEV in the United States is critical to the classification of the virus and the potential public health risks associated with it. Furthermore, the lack of efficient cell culture systems for HEV emphasizes the importance for the identification of new animal models in HEV research. Because of their size and ease of keeping and the availability of reagents, rabbits represent a potential new and useful animal model for HEV research. The zoonotic ability of HEV is now documented and the search for possible new animal reservoirs and routes of foodborne transmission is of paramount importance.

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Chapter 1: Hepatitis E Virus - Literature Review

BACKGROUND

General History

An outbreak of water-borne acute hepatitis in New Delhi, India in 1955 was found to have resulted from a new viral etiological agent that was originally known as non-A, non-B hepatitis virus (75, 159). Spherical, 27-30nm virus-like particles (VLPs) were identified using immune electron microscopy to examine the feces of a volunteer who ingested fecal matter from patients with non-A, non-B hepatitis. In 1990, the novel virus responsible for the non-A, non-B enteric hepatitis epidemics was identified and named hepatitis E virus (HEV) (128). HEV infections were initially detected in developing countries with sub-optimal sanitation practices and were diagnosed by exclusion in absence of acute markers for hepatitis A virus (HAV), hepatitis B virus (HBV) and other causes of acute hepatitis (159). The entire genome of HEV was sequenced in 1991 (144).

Initially, diagnoses of HEV cases were by exclusion only after other known causes of acute hepatitis were ruled out. Because of increased awareness of HEV and the advancement of diagnostic assays, HEV has been recognized as being an important human pathogen found in most parts of the world. It is known to be associated with individual acute and chronic hepatitis cases as well as the classic, acute explosive outbreak presentation. Many animals have also been found to carry HEV after first being discovered in commercial pigs in 1997, and much research is currently being done to investigate the zoonotic potential of HEV (104).

Epidemiology

Hepatitis E is endemic in many developing countries. Epidemic outbreaks of genotypes 1 and 2 viruses have been reported from many parts of Asia, Africa, and Mexico (3, 22, 31, 76).

However, it is now known that HEV is no longer confined to developing countries, and an increased incidence of sporadic cases of HEV has been reported in industrialized countries. Among industrialized countries, HEV has been reported in the US, Japan, and many countries within the European Union (39, 80, 107, 133).

A recent publication in the National Health and Nutrition Evaluation Survey (NHANES) determined that the seroprevalence of HEV in the general population in the United States is only about one-third as high as previously reported (27). A total of 8,814 individuals were included in this analysis. The weighted national Seroprevalence of HEV was 6% (95% CI 5.1%-6.9%). According to the report, about 0.5% of those with HEV had evidence of recent exposure (IgM positive). In the univariate analyses, factors associated with HEV seropositivity were increasing age (P-value <0.001), birth outside of the US, Hispanic race, and “meat” consumption (>10 times/month). No significant associations with low socio-economic status, water source, or level of education were observed (27).

Cases of acute hepatitis E account for a large proportion of cases of acute liver disease in developing countries, with smaller (although unknown) proportions in Europe and the United States. In industrialized countries, individual cases and small outbreaks have been linked to exposure to pigs and consumption of undercooked pork or wild game (41, 82, 91, 93, 145, 148, 161, 163). Recent studies have found that HEV RNA was present in samples when testing pig liver and sausage from commercial groceries in Europe and the United States, (21, 41). Furthermore, laboratory analysis showed the presence of infectious HEV in rare to medium-rare meat (42). Case reports have also linked HEV to consumption of shellfish and blood transfusions, but the overall rate of these risk factors among unselected patients is low (48, 79, 92). Furthermore, secondary spread from this zoonotic transmission is rare (55).

Taxonomy and Classification

HEV is a small, nonenveloped virus with a single-stranded positive-sense RNA genome that is approximately 7.2 kb in length and contains three partially overlapping open reading frames (ORFs) that are flanked by short 5' and 3' nontranslated regions (14, 38, 95). HEV was initially classified within the family *Caliciviridae* on the basis of similar morphology, physiochemical characteristics, and genome organization resemblances (95). However, since the initial classification, there has been a lack of significant sequence homology and evidence of major differences in genomic organization from that of caliciviruses. It has since been reclassified into the only genus, *Hepevirus*, within the *Hepeviridae* family (32, 95).

Two major species of HEV have been recognized. Mammalian HEV has been documented to cause acute hepatitis in humans and has known animal reservoirs (96), and avian HEV has been associated with hepatitis-splenomegaly syndrome in chickens (119). There are currently four recognized geographically distinct HEV genotypes within the genus *Hepevirus*. These four genotypes fall into two major groups: genotypes 1 and 2 are human viruses that have been identified as causing epidemic hepatitis and are associated with waterborne and fecal-oral transmission; while genotypes 3 and 4 have been identified in humans and other animal species (96, 97). Genotype 1 is made up of human HEV strains from Asia, Africa, and recently, Cuba. Genotype 2 is made up of human HEV strains isolated from Mexico and Africa. Genotype 3 includes strains from sporadic cases of human HEV in industrialized countries, swine HEV strains from developing and industrialized countries, and strains isolated from other animals including domestic and wild pigs, deer, mongoose, ferrets, and rabbits (94, 96, 98). Genotype 4 is comprised of human and swine HEV strains isolated from Asia, specifically from China, Japan, Taiwan, and Vietnam (94, 96, 97). Recently, two putative new HEV genotypes were identified from wild boars in Japan and rats in Germany (66, 67, 143). There is cross-

neutralization among all HEV genotypes, indicating that they belong to a single serotype, despite clinical and epidemiologic differences.

Avian HEV from chickens shares approximately 50% nucleotide sequence identity with mammalian strains and is classified as a separate species in the family *Hepeviridae* (32, 95). There are currently three genotypes of avian HEV that have been identified from chickens throughout the world (9, 95, 97, 120). Because of the extensive sequence variation between avian and mammalian strains of HEV, it has been proposed to re-classify avian HEV as a new genus within the *Hepeviridae* family (97).

The currently available cell culture systems for HEV are inefficient, however, they do support the full replication cycle of the virus (116). As a result, these systems allow an opportunity to understand the mechanisms responsible for cell entry, genomic replication, virion morphogenesis, and viral egress *in vitro* (2). Additionally, the construction of infectious cDNA clones and replicons of HEV has enabled the study of individual HEV genes (13, 35, 47, 60), and the development of small animal models has allowed for easier study of HEV replication and pathogenesis in homologous animal model systems (10, 19, 158).

MOLECULAR VIROLOGY

Genome Organization

Open Reading Frame 1 (ORF1)

ORF 1 is the largest of the three open reading frames in the HEV genome and encodes for the nonstructural proteins. ORF1 consists of approximately 1700 amino acids and contains several putative functional motifs and domains such as methyltransferase (MeT), papain-like cysteine protease (PCP), RNA helicase, and RNA dependent RNA polymerase (RdRp) (14, 37, 38). The presence of the MeT motif in ORF1 suggests that HEV has a capped RNA genome

(37). The viral RdRp contains eight conserved motifs (I-VIII) that appear to be functionally similar to those of other positive-sense RNA viruses (77). The GDD motif in RdRp was reported to be important for replication (34). Additionally, the RdRp seems to be localized to the endoplasmic reticulum (ER), which suggests that the ER is likely involved in HEV replication (125). Two stem-loop structures at the 3' NCR and polyA tract were necessary for RdRp binding during genome replication (14, 37).

A hypervariable region (HVR) was identified in the ORF 1 of HEV. The HVR overlaps the pro-rich sequence between the N terminus of the X domain and the C-terminus of the putative PCP domain (14, 37, 78). As the name suggests, there is much variation in both length and sequence in the HVR of different strains of HEV. The HVR has been shown to be dispensable for viral infectivity, however is critical for viral replication (122). Additionally, the HVR sequences appear to be functionally interchangeable between different HEV genotypes with respect to replication and infectivity *in vitro* (122).

Open Reading Frame 2 (ORF2)

The ORF2 of HEV encodes a capsid protein made up of approximately 660 amino acids and encapsidates the viral RNA genome (38, 97). It is well documented that the ORF2 capsid protein is involved in the assembly of the HEV virion and its interaction with host cells (14). It is proposed that the ORF2 protein contains a ER localization signal in its N terminus (139). The ORF2 protein also bound the 76-nucleotide region at the 5' end of the HEV genome, which supports its capsid encapsidation function, and may play a role in viral assembly (140). Additionally, the capsid protein interacts with host cells and binds to heparin sulfate proteoglycans (HSPGs) and chaperone proteins heat shock protein 90 (HSP90) and glucose-

regulated protein 78 (Grp78) (53, 70). HSPGs are believed to serve as attachment receptors for HEV to enter into host cells, while the chaperone proteins assist with intracellular transport (70).

The capsid protein of HEV is immunogenic and has been shown to be targeted by neutralizing antibodies (14). There is strong evidence for the existence of only one serotype of HEV and some degree of cross-reactivity between the capsid proteins of all strains. Western blot analysis showed that a recombinant avian HEV capsid protein reacted both with antibodies raised against genotype 1 human HEV and antibodies raised against genotype 3 swine and human HEVs. Additionally, convalescent sera from avian HEV experimentally infected chickens reacted with the recombinant capsid proteins from the genotype 3 swine HEV and genotype 1 human HEV (50). Convalescent serum collected from animals that were infected with any of the four mammalian genotypes of HEV were all able to neutralize genotype 1 HEV (33).

Open Reading Frame 3 (ORF3)

The ORF3 of HEV encodes a small cytoskeleton-associated phosphoprotein that consists of approximately 120 amino acids and is translated from the bicistronic subgenomic RNA (14, 47). ORF 3 overlaps ORF2 by approximately 300 nucleotides at its 3' end, but does not overlap ORF1 (61). While ORF3 was dispensable for replication *in vitro*, it is required for both infection of rhesus macaques and the release of virions from infected cells (35, 36, 46, 160). The ORF3 protein is thought to regulate the host cell environment through its interaction with various intracellular pathways, which in turn promote cell survival. It activates the extracellular regulated kinase (ERK) by binding and inhibiting its Erk-specific MAPK phosphatase (74). Additionally, the ORF3 protein might act as an adaptor to link intracellular transduction pathways, and this might promote HEV replication and assembly (111). It has also been shown that the ORF3 protein localizes to early and recycling endosomes and delays post-internalization trafficking of

epidermal growth factor receptor (EGFR). This prolongs endomembrane signaling and likely promotes cell survival (17). Another effect of this is reduced nuclear translocation of pSTAT3 and attenuation of the acute phase response. Thus, ORF3 might reduce the host inflammatory response, further creating an environment favourable for viral replication (14, 17).

HOSTS & RESERVOIRS, CROSS-SPECIES TRANSMISSION, AND ZOOZOSES

Hepatitis E Virus is unique from all other existing human hepatitis viruses in that it has been linked to animal reservoirs, beginning with its isolation from swine in 1997 (104). Genetic identification of HEV strains from other animal hosts and the demonstration of cross-species transmission of the virus by some strains have broadened the host range and genetic diversity of the virus. Thus far, HEV RNA has been isolated from humans, pigs, wild boars, chickens, rabbits, deer, rats, ferrets, mongoose, bats, fish, and moose (52, 67, 84, 96, 99, 104, 124, 148, 163, 165). Several other species of animals have been found to have high prevalence of seropositivity for anti-HEV antibodies, however, the virus has not yet been isolated from them. Because of this, it is likely that further study of the host range of HEV will lead to the discovery of even more animal hosts and reservoirs of the virus. Beyond its wide host range, low mortality and subclinical infection in its hosts, it is unclear exactly how HEV is maintained within populations of people or herds of animals. The clinical and pathological effects of HEV infection in its animal hosts have not been extensively studied thus far. The histopathologic lesions appear to be similar among animals naturally or experimentally infected by different strains of HEV, but the manifestation of clinical diseases varies among species. There are no pathognomonic lesions associated with HEV infection that differentiate it from other hepatotropic viruses. The fecal-oral route is implicated as the primary mode of transmission of HEV among humans and animals alike, and is also the probable route of infection between different species of animals (155).

There has been increasing evidence of infection due to ingestion of HEV-positive animal by-products, which is likely the main route of zoonotic infection from HEV infected animals to humans (8, 21, 108, 117, 126, 149, 155, 161).

Hosts and Reservoirs of HEV

Humans

Hepatitis E in humans is endemic in both industrialized and developing countries worldwide. Epidemics, however, only occur in the developing countries of Asia, Africa, and in Mexico. The epidemics in developing countries are usually associated with HEV-contaminated drinking water due to poor sanitation practices, whereas the sporadic cases in industrialized and developing countries are associated with contaminated animal meats, shellfish, and direct contact with infected animals. Humans are known to be infected by genotypes 1 and 2 HEV that are restricted to human hosts, and also genotypes 3 and 4 that have an expanded host range, have been isolated from several animal species, and have also been known to cause zoonotic transmission (96, 97).

In the human host, acute HEV has an incubation period of 3 to 8 weeks, a short prodromal phase, and a symptomatic phase that lasts from days to several weeks. Most cases of HEV are self-limiting and few result in chronic hepatitis (96, 97). In large surveys of acute liver failure in the United States, cases resulting from HEV infection were rare, accounting for less than 1% of adult cases (17).

In immunocompetent people, HEV infection is usually self-limiting with no apparent clinical disease. Clinical disease due to HEV infection is rarely diagnosed in industrialized countries, and most cases of HEV infection in industrialized countries occur in people who have traveled to regions where the disease is endemic. The HEV target population is typically young

to middle aged adults, 15 to 40 years of age. It causes a less than 1% mortality among immunocompetent people, however the mortality can reach up to 25% in pregnant women (114).

Typical symptoms of HEV infection in humans are jaundice, dark urine, anorexia, enlarged livers, elevated ALT levels, abdominal pain, and tenderness sometimes accompanied by nausea, vomiting, and fever (17, 110). The disease can range in severity from sub-clinical to fulminant during pregnancy. Common complications during pregnancy include: death of the mother and fetus, abortion, premature delivery, or death of the infant soon after parturition (110, 114). A study looking at histopathology in 11 patients with acute hepatitis E showed lesions in all cases with marked necroinflammatory activity in 9 patients. Characteristic pathological signs of acute HEV infection were severe intralobular necrosis, polymorph inflammation, and acute cholangitis with numerous neutrophils (110, 121).

Originally, HEV was thought to resemble hepatitis A virus (HAV) and only cause acute, self-limiting infections, unlike hepatitis B and C viruses. Recently, however, there have been many reported cases of chronic hepatitis E. Chronic infection has been identified almost exclusively among immunocompromised patients, including HIV-infected people, chemotherapy patients, and organ transplant recipients (17, 97). In these cases, HEV RNA has been detected in serum and stool and has persisted for years (97).

Under experimental conditions, human strains of HEV have successfully infected several species of animals. Genotypes 3 and 4 strains of human HEV have been able to infect pigs (43, 158), however, genotypes 1 and 2 human HEV appear to have a more limited host range and are restricted to humans, because attempts to experimentally infect pigs with genotypes 1 and 2 human HEV were unsuccessful (101). To further support this, no rabbits inoculated with genotype 1 human HEV were successfully infected (87). Lambs and Wistar rats were also

reportedly infected by human HEV isolates (88, 153), however this has not yet been independently confirmed. In a subsequent study by a different group, laboratory rats inoculated with human HEV genotypes 1, 2, 3 that can infect primates or pigs, failed to produce any evidence of infection (123). There has been no evidence of natural cross-species infection of animals with human strains of HEV outside of laboratory settings.

Domestic Pigs

Swine HEV was the first animal strain of HEV to be identified and characterized from pigs in the United States (104). In this study, adult pigs were found to be seropositive for anti-HEV antibodies, suggesting exposure to HEV. A prospective study was then performed and led to the eventual discovery of a novel virus distantly related to human HEV (104). Since the original identification, swine HEV has been detected in domestic pigs from nearly all major pork-producing countries throughout the world (97, 115). Antibodies to HEV have been detected in many industrialized countries, including the United States, Canada, Korea, Taiwan, and Australia (16, 56, 100, 104). Antibodies to HEV have also been detected from pigs in countries where HEV is endemic, such as Nepal and China (20, 100). To date, pigs have been identified as the major reservoir of the two zoonotic genotypes of HEV, genotypes 3 and 4 (96). HEV infection seems to be widespread in swine farms from both developing and industrialized countries (96).

The virus generally infects pigs of 2 to 4 months of age, resulting in transient viremia for 1 to 2 weeks and fecal virus shedding for up to 7 weeks. The infected pigs are subclinical and generally have a transient viremia lasting for 1 to 2 weeks, and fecal virus shedding lasting for approximately 3 to 7 weeks (104, 142). The transmission route for HEV in pigs is thought to be primarily fecal-oral and virus-containing feces appears to be the principal source of the virus. It

is believed that pigs acquire infection through direct contact with infected pigs or through ingestion of feces-contaminated feed or water (98). Under experimental conditions, however, infection of pigs with HEV via the oral route of inoculation has been difficult, even though pigs can be readily infected via the intravenous route of inoculation. The mechanism of how HEV is maintained in swine herds is unknown (96).

Swine HEV is thought to cause only subclinical infection in domestic pigs. In pigs naturally infected by swine HEV, clinical illness has not been observed and all infections have been subclinical (98). In a prospective study of swine HEV infection in a commercial farm, 4 piglets at an early stage of natural infection were necropsied (98, 104). Gross lesions were not apparent in 19 different tissues or organs examined during necropsy. Microscopically, all 4 piglets necropsied had evidence of hepatitis characterized by mild to moderate multifocal and periportal lymphoplasmacytic hepatitis with mild focal hepatocellular necrosis. Additionally, all of the piglets had lymphoplasmacytic enteritis and three of them also had multifocal lymphoplasmacytic interstitial nephritis (104). In a study that experimentally infected pigs with swine HEV, there was no observation of clinical disease or elevation of liver enzymes or bilirubin. In these pigs, mild-to-moderate multifocal lymphoplasmacytic hepatitis and focal hepatocellular necrosis were observed in infected pigs. HEV RNA was detectable in feces, liver tissue, and bile of infected pigs from 3 to 27 days post-inoculation (49).

Genotypes 3 and 4 swine HEV are now recognized as zoonotic viruses, with domestic pigs as the primary reservoir (96, 97). Direct human contact with infected pigs poses a risk for HEV infection. Pig handlers such as pig farmers and swine veterinarians are at increased risk of HEV infection. For example, swine veterinarians in the United States were shown to be 1.51 more likely to have anti-HEV antibodies than age- and geography-matched normal blood donors

(105). Additionally, individuals from traditionally major swine-producing regions are more likely to be seropositive for antibodies to HEV than those from non-swine regions (105).

Another risk factor human infection by swine HEV is the consumption of undercooked pork. It has been demonstrated that contaminating virus was completely inactivated when HEV-contaminated pig livers were fully cooked, however, cooking the liver to a temperature equivalent to medium-rare conditions in a restaurant did not inactivate the virus (41). Sporadic cases of acute hepatitis E have been linked to the consumption of raw or undercooked pig livers and pork (82, 92, 93). Other implicated routes of zoonotic transmission of swine HEV to humans include transplantation of genotype 3 and 4-infected organs or blood and xenotransplantation of infected pig livers into human recipients (72, 98).

Aside from humans, genotypes 3 and 4 swine HEV have been used under experimental conditions to infect several species of animals. Genotypes 3 and 4 strains of swine HEV can cross species barriers and infect both rhesus macaques and chimpanzees (4, 102). Genotype 4 swine HEV has also been shown to be infectious in rabbits, albeit an inefficient infection (87).

Wild Boar

Wild boars are indigenous in countries worldwide and have been shown to be infected with HEV (94). Human habitation changes from rural to suburban areas, increased agricultural use of lands, deforestation, recreational hunting, and consumption of wild boar meats have increased the chances of contact exposure of wild boars to humans (94, 103). The HEV strains identified in wild boars worldwide appear to belong primarily to genotype 3, although strains belonging to genotype 4 as well as a putative new genotype have also been detected in wild boars (96, 132, 136).

HEV RNA and anti-HEV antibodies have been detected in wild boars from many countries worldwide including: Japan, Germany, Italy, Spain, the Netherlands, and Australia (16, 26, 69, 90, 106, 129). From these reports, HEV seropositivity ranges from 17 to 42%, with a RNA detection rate ranging from 3-25% in the different regions. A full-length sequence of HEV amplified from a wild boar in Japan is nearly identical to a virus recovered from a deer in the same forest and to four human patients who consumed deer meats and developed hepatitis E (141).

No studies have investigated the clinical disease and pathologic effects of HEV in wild boars. However, because of the close relatedness of wild boar HEV to swine HEV, and of boars to domestic pigs, it is likely that HEV in boars is also subclinical and that boars are a natural reservoir of the virus. Like domestic pigs, the genotypes 3 and 4 HEV from wild boars infect humans (94, 96). Recreational hunting of wild boars and the consumption of wild boar meat have provided a route of transmission of HEV from wild boars to humans (136). Because of its nearly identical genomic sequence to a strain of HEV recovered from deer in the same forest in Japan, it has been presumed that the boar must be a natural reservoir of HEV while deer are likely an occasional reservoir, infected from boars in the habitat of these animals (141). Beyond humans and deer, the host range of wild boar HEV is unknown, however due to its close similarity to other genotype 3 viruses and with its ancestor, the domestic pig, it likely can infect other mammals.

Chickens

Avian hepatitis E virus was genetically identified from chickens with hepatitis-splenomegaly syndrome (HSS) in the United States (51). Avian HEV shares approximately 80% nucleotide sequence identity with the big liver and spleen disease virus (BLSV) from chickens in

Australia, suggesting that BLS in Australia and HSS in the United States are genetically related. BLSV causes decreased egg production and a slight increase in mortality in commercial broiler breeder flocks. Avian HEV shares an approximate 60% nucleotide sequence identity and common antigenic epitopes in the capsid protein with human HEVs and three genotypes of avian HEV have currently been identified from chickens worldwide (9, 50, 89).

In the United States, HEV infection in chickens is enzootic, with approximately 71% of chicken flocks and 30% of chickens testing seropositive for avian HEV antibodies (58, 137). Avian HEV appears to be an age-dependent infection, with approximately 17% of chickens less than 18 weeks of age and 36% of adult chickens testing positive for avian HEV antibodies (58). It appears that avian HEV is readily transmitted within and between chicken flocks and infection has been experimentally reproduced via the oro-nasal route of inoculation in chickens. This suggests that the route of transmission is most likely fecal-oral, but other routes of transmission cannot be ruled out (10, 94).

Avian HEV infection in chickens is usually subclinical; with relatively low morbidity and mortality (58, 94, 137). Infected chickens can display higher than normal mortality in broiler breeder hens and laying hens of 30-72 weeks of age, with the highest incidence occurring between 40-50 weeks of age. Egg production has been shown to drop by 20% (58, 94). At necropsy, dead chickens associated with avian HEV infection tend to have regressive ovaries, red fluid in the abdomen, and enlarged livers and spleens. Livers are enlarged due to hemorrhaging and some have subcapsular hematomas. Spleens from affected birds are mild to severely enlarged (10, 94). In chickens experimentally infected by avian HEV, subcapsular hemorrhages and enlarged livers were observed in approximately 25% of the infected chickens.

Microscopic hepatitis lesions in experimentally infected chickens are characterized by lymphocytic periphlebitis and phlebitis in the livers (10, 94).

Like the genotypes 3 and 4 swine HEV, avian HEV can also infect across species barriers. Avian HEV from a chicken was shown to successfully infect turkeys (138). A study attempted to determine whether avian HEV also infects non-human primates by experimentally inoculating two rhesus macaques with avian HEV. Evidence of virus infection was not observed in the inoculated monkeys, as there was no seroconversion, viremia, fecal virus shedding, or serum liver enzyme elevation (137). The results from this study suggest that avian HEV is genetically related to, but distinct from, human and swine HEV. Unlike swine HEV, avian HEV appears to have a more limited host range is unlikely to be able to infect human and non-human primates (59). The ability of avian HEV to infect other animals is unknown.

Rabbits

The first evidence of HEV in rabbits was from farmed rabbits in Gansu Province, China. In this study, three full-length genomic sequences of the rabbit HEV were determined and suggested that rabbit HEV was a distant member of genotype 3 HEV that are known to be zoonotic (165). A study in 2011 isolated HEV from farmed rabbits in Virginia, USA and confirmed that rabbit HEV was indeed closely related to genotype 3 viruses (23). This study determined HEV RNA was detected in 14 (16%) of 85 serum samples and 13 (15%) of 85 fecal samples. Antibodies against HEV were detected in 31 (36%) of 85 serum samples. This data indicates that similar to swine HEV in pigs and avian HEV in chickens, rabbit HEV is also widespread in the rabbit population in the United States (23). Rabbit HEV has also been subsequently isolated from wild and farmed rabbits in France (62).

Rabbit HEV infection in rabbits does not appear to cause overt clinical disease, raising the possibility that rabbits are a natural reservoir of the virus. In the first study to look at the pathogenic effects of rabbit HEV infection in rabbits, researchers experimentally infected rabbits with several strains of rabbit HEV. Serum ALT levels, indicative of recent liver damage and suggestive of acute infection, were elevated in HEV infected rabbits. In the same study, local hepatocellular necrosis was also observed in a rabbit infected with rabbit HEV (87). More studies are warranted to investigate the pathogenicity of rabbit HEV in the rabbit model.

Under experimental conditions, rabbit HEV has been successfully transmitted to pigs, the natural reservoir of many genotype 3 and 4 viruses (24), demonstrating the ability of cross-species infection by rabbit HEV. Rabbit HEV has also recently been shown to be able to infect cynomolgus macaques (86). This, coupled with its close genetic relatedness to zoonotic genotype 3 viruses, indicates that human infection by rabbit HEV is a possibility. Further studies are warranted to investigate the zoonotic potential and host range of rabbit HEV.

Deer

Anti-HEV antibodies were detected by ELISA in approximately 3% of the sitka deer and 35% of Yezo deer sampled in Japan, and 5% of red deer sampled in the Netherlands (129, 150). The full-length genome of a strain of HEV from sitka deer in Japan was determined and sequence analysis showed that the deer HEV belongs to genotype 3. Subsequently, genotype 3 strains of HEV were identified from roe deer in Hungary (127). This indicates that HEV infection in deer is widespread and likely a cause of HEV infection among humans and deer worldwide.

Clinical and pathologic effects of HEV infection on wild deer has not been investigated, although it is likely that, similar to other strains of genotype 3 HEV, deer HEV causes only

subclinical infection in its host. Transmission of HEV from deer to humans via the consumption of contaminated deer meats has been reported, so deer HEV is considered zoonotic (146, 147). Due to its close relatedness to wild boar HEV from Japan, it is possible that deer HEV may naturally infect other animals (141). The cross-species transmission of deer HEV to other species of animals has not yet been investigated.

Rats

Rats have long been implicated as potential reservoirs of HEV. They are particularly interesting as a potential source of human infections, because while they are not human food, they are ubiquitous and in close contact with humans globally. HEV have been detected in the serum of various species of rats including Norway (*Rattus norvegicus*), black (*Rattus rattus*), and cotton (*Sigmodon hispidus*) rats (6, 40, 68, 94). Strains of HEV have also now been isolated from several species of rats (65, 67, 123). The rat HEV shared only approximately 60% and 50% sequence identity with other mammalian HEV and avian HEV, respectively, and thus belongs to a putative new genotype within the *Orthohepevirus* genus (94).

A recent study looked at the liver enzyme and histopathologic changes associated with the experimental infection of laboratory rats with rat HEV. All 6 experimentally infected rats seroconverted post-infection, yet liver enzyme levels varied considerably, but were not temporally associated with seroconversion (123). So, the study concluded that these infections were biochemically inapparent infections and there were no clinical manifestations of disease. For the histological evaluation, two HEV-infected rats and two negative controls were examined under code for histological evidence of hepatitis. The 2 uninfected rats had essentially normal livers. The two HEV infected rats developed parenchymal foci of necrosis and aggregates of lymphocytes and Kupffer cells in hepatitis lobules and had mild portal inflammation (123). In

conclusion, rat HEV has been shown to cause minimal hepatitis in experimentally infected animals; liver enzyme levels seldom increase above baseline levels, and histopathologic lesions during acute infections, although present, were minimal and not associated with clinical disease.

Thus far, the public health implications of rat HEV are unknown. Laboratory rats inoculated with mammalian HEV genotypes 1, 2, 3 that can infect primates or pigs, failed to produce any evidence of infection (123). However, strains of HEV belonging to the genotype 3 have recently been identified from rats in the United States, suggesting that rats might be naturally susceptible to some genotype 3 strains. More research needs to be done in this area to determine the public health risk associated with these strains of HEV (81, 123).

Ferrets

A unique strain of HEV was genetically identified from ferrets in the Netherlands (124). Of the known strains of HEV, the ferret HEV was most closely related to rat HEV. Phylogenetic analysis revealed that the ferret HEV was distinct from the known genotype 1-4 mammalian HEV in the proposed *Orthohepevirus* genus and clustered with the putative new genotype of rat HEV. Sequence identity with HEV genotypes 1-4 and rabbit and avian HEVs ranged from 54.5 to 60.5% (124). There has only been one study that has reported HEV in ferrets. As a result, there is no information regarding the clinical disease this virus may cause because the samples were obtained from household pet ferrets that did not show any overt clinical signs (124). Because of its lack of nucleotide identity shared with genotypes 1-4 it is unlikely that ferret HEV poses a risk of zoonotic transmission to humans. The potential of ferret HEV to be transmitted to other animals has not yet been investigated.

Mongoose

Approximately 8 to 21% of wild mongooses tested in Japan were seropositive for IgG anti-HEV (83). A subsequent study was able to isolate genotype 3 strains of HEV from wild mongooses (112). No studies have yet been done to look at the clinical and pathologic effects of HEV infection in mongooses. There were no observed clinical signs of infection in the wild mongooses that were sampled in the studies to date (83). It is unknown whether mongoose HEV can infect humans, but other genotype 3 strains of HEV are known to be zoonotic. Further studies are warranted to investigate how widespread HEV infection is among mongooses in other regions and also the zoonotic and cross-species potential of the virus, as it is so closely related to other zoonotic genotype 3 strains.

Bats

Bats have proven to be highly efficient indicators of mammalian virus diversity, most likely due to their exceptionally large social group sizes that promote the acquisition and maintenance of viruses (12, 28). A recent study investigated a globally representative sample from 85 different bat species from 5 continents, totaling over 3,500 specimens (30). Novel strains of HEV were identified from Western African, Central American, and European bats. The virus was isolated from five bat species of three different families, representing all of the major lineages of bats. The bat HEV forms a novel phylogenetic clad, the most divergent strains thus far, belonging to a separate genus within the family *Hepeviridae* (30).

Due to the subclinical nature of HEV in bats, it is likely that bats are a natural reservoir of the virus. No studies have looked at the pathologic effects of bat HEV on bats thus far. A recent study that screened humans that were considered to be at an increased risk of exposure to bats for

bat HEV RNA did not yield any evidence of HEV transmission from bats to humans (30). This is not surprising considering the high dissimilarity between bat HEV and other known strains of HEV. Even though humans in parts of the world consume bats for food, it is unlikely that bat hepeviruses would easily transmit to humans (30). As discussed above, the viruses isolated from pigs, wild boars, and deer that have been demonstrated to be zoonotic closely related to human viruses. Neither avian nor rat hepeviruses were transmissible to primates experimentally, and they are more closely related to human viruses than the bat viruses (59, 123). Bats, therefore, do not seem to constitute reservoirs of mammalian hepeviruses and based on current data do not seem to currently pose a risk of zoonotic transmission to humans or other mammals

Fish

In 1991, a novel RNA virus not associated with disease was isolated from trout in California. The virus was subsequently isolated from several species of trout throughout the United States and termed the cutthroat trout virus (CTV) (7, 54). In 2011, the full-length genome sequence of CTV was determined and it was concluded that it is a novel member of the family *Hepeviridae*, the first strain in this family from an aquatic animal (7). The virus only shares approximately 13 to 27% amino acid sequence identity with the proposed avian genera *Orthohepevirus* and *Avihepevirus*, so it likely belongs to another new proposed genus *Piscihepevirus*.

When grown in the Chinook salmon embryo cell line (CHSE-214), the virus produced a slow, focal type of cytopathic effect (CPE) that did not result in the destruction of the entire monolayer. Thus far, experimental infection of several species of trout and salmon with CTV has not been associated with mortality or microscopic pathology (54). This is in contrast with hepatitis E virus infections of humans that has been shown to lead to outcomes ranging from sub-

acute to fulminant forms of disease, often associated with hepatic lesions and mortality, particularly among women during the later stages of pregnancy (7, 63, 64).

The zoonotic and cross-species transmission potential of this new strain of the family *Hepeviridae* has not yet been examined. Due to its low nucleotide identity with other members of the virus family, particularly HEV genotypes 1-4 that have been shown to infect humans and other mammals, it is unlikely that this virus poses a threat to human or animal health.

Horses

The first study to detect anti-HEV antibodies in the serum in horses was in Egypt. Horses are widely used throughout rural and suburban Egypt for pulling carts and they live and work in close contact with humans. This study screened the serum of 200 workhorses in suburban Cairo by ELISA for the presence of IgG anti-HEV antibody. Of these, 26 (13%) of the serum samples tested positive; with 12 of these having relatively high OD values (2-4.5 times greater than the cut-off value) (130). From the 200 total serum samples, 100 samples were processed for viral RNA detection by RT-PCR. Four of these serum samples were PCR-positive for HEV viral genome. All of these positive samples were from samples that tested negative by ELISA. The mean age of the viremic animals was 0.3 years, and all 4 positive samples were from horses under 1 year of age. After sequencing, the 4 sequences returned from the positive horses shared 97-100% nucleotide identity with the sequences of human HEV isolates recovered from Egypt previously (130, 151). Because of the close-relatedness of these sequences to the Egyptian human HEV isolates, it indicates that these viruses likely originated from a common ancestor, and raises the possibility that horses are either accidental hosts or reservoirs for HEV, and that they are a possible source of infection to humans (130). There has been no published data on prevalence of anti-HEV antibodies or HEV RNA in samples from horses in other geographical

locations, any clinical manifestations or pathogenicity of hepatitis E infection in horses, or any demonstrated cases of cross-species transmission of the virus.

Other potential animal hosts of HEV

Several animal species have demonstrated a high prevalence of positivity for anti-HEV antibodies in serum, but the virus has not yet been successfully isolated from them. Antibodies to HEV have been detected in cattle from different countries. Anti-HEV IgG were detected from 4.4 to 6.9% of cattle in India, and 6 to 93% of cattle in China (6, 44, 157, 164). A 189-bp sequence of HEV was reportedly amplified from the fecal samples of eight cows in China, and the bovine HEV appears to be a genotype 4, however attempts to replicate these findings have been unsuccessful (57).

Serological evidence of HEV infection in sheep has been reported in China and Spain. Approximately 10-12% of the sheep sampled in China and 2% of the sheep sampled in Spain were positive for IgG anti-HEV (18, 120, 162). A 189-bp sequence of HEV was amplified from six sheep fecal samples in China by the same laboratory that reported the sequence of HEV in cattle, and the sheep HEV also appears to be a genotype 4 (156). Like the genotype 4 HEV sequences reportedly amplified from cows, sheep HEV requires further independent confirmation.

Serological evidence of infection has been reported in several other animal species such as: dogs, cats, goats, and non-human primates (5, 45, 85, 109, 131, 152, 154). This indicates that these species have been exposed to HEV. Further studies conceivably isolate the virus from these species, thus expanding the known host range of the virus. More research into the expanding host

range and zoonotic and cross-species transmission potential of HEV will likely illuminate more natural reservoirs of the virus.

Environmental Safety Concerns

Contamination of water by HEV from human and animal wastes can lead to subsequent contamination of food and produce, thus leading to foodborne transmission of the virus (155). Historically, waterborne epidemics are the characteristic of genotypes 1 and 2 hepatitis E outbreaks in humans in regions where sanitation conditions are poor (1, 155). Untreated sewage water and contaminated well or river water used for washing and drinking purposes remain the primary sources of HEV transmission in developing countries. In industrialized countries with good sanitation conditions and water treatment facilities, outbreaks of water-borne HEV transmission are rare (1). However, the existence of numerous zoonotic strains of HEV from various animal species implies that land application and runoffs of infected animal manure could contaminate irrigation or coastal water, posing a risk of foodborne and waterborne HEV transmission (1, 11, 155).

HEV replicates in the liver and gastrointestinal tract of infected humans and animals, and the infected host excretes large amounts of infectious virus in the feces (158). This poses a concern of environmental contamination and food safety. HEV has been isolated from swine manure and wastewater associated with hog operations, and in concrete lagoons of swine manure storage facilities in the United States (155). HEV RNA has been isolated from shellfish in the United Kingdom, Korea, and Thailand (25, 29, 113, 155). Consumption of contaminated shellfish has been implicated in sporadic cases of acute hepatitis E (15). Additionally; an outbreak of hepatitis E on a cruise ship was linked to the consumption of shellfish while on board (25). These cases have confirmed suspicions of environmental contamination by HEV.

CONTROL AND PREVENTION

Transmission dynamics of HEV within population groups are not yet fully understood. Because of the nature of how the virus is spread (through the fecal-oral route and contaminated meat), the fact that it is endemic in many developing and industrialized countries, and that it causes only subclinical disease in many of its animal hosts, elimination of the disease with in human and animal populations is highly unlikely in the foreseeable future. Aside from letting the disease run its course and using standard prophylaxis as treatment, the most realistic control and prevention strategies for humans are properly washing and cooking all food that could be contaminated by the virus prior to consumption, ensuring adequate hygiene in endemic areas or if working in high risk jobs (veterinarians, pig herders, sewage workers, etc), and the use of antivirals and vaccines.

Treatment

Treatment of acute HEV is not usually necessary, as the natural course of the infection is self-limiting with spontaneous recovery. Typically, only immunocompromised patients require treatment, but older men with underlying liver disease are also vulnerable to severe and even fatal acute infections for which treatment is oftentimes suggested (118, 134). There are reports of successful treatment of both acute and chronic genotype 3 HEV with regimens including pegylated interferon and/or ribavirin, especially in patients with underlying chronic liver disease (71, 73, 118, 134). Ribavirin has been shown to inhibit the viral RNA replication and induce a sustained virological response in chronically infected patients (73). In the case of HEV-infected organ transplant recipients, immunosuppressive drugs should first be reduced. If infection persists, 3 months of ribavirin is a typical treatment strategy, since interferon has been shown to cause graft rejection in heart, lung, and kidney transplant recipients (71, 73). There are no current

reports of treatment of genotype 1 HEV in humans, treatment of pregnant women, or any treatment of animals due to the cost and generally subclinical nature of the disease in non-human hosts.

Vaccine

Under experimental conditions, it has been shown that when animals are infected with acute HEV and then re-challenged with HEV, they are protected against reinfection. Additionally, when plasma from an infected animal was infused into a naïve animal, the recipient acquired protection (134). There has also been some epidemiologic data showing that in areas of the world where HEV is endemic, people that have had prior exposure to the disease have some level of protection against infection. There is only one serotype of HEV, so there is serologic cross reactivity among all four recognized viral genotypes (24). As a result, antibodies developed from one particular genotype protect against all genotypes. The result of this information has led to the development of two recombinant vaccines for HEV.

The safety and efficacy of the first HEV recombinant protein vaccine have already been evaluated in a phase II clinical trial. It proved safe and immunogenic in young men (mean age of 25.2 years) and provided 95% protection against hepatitis E in Nepal, where only genotype 1 hepatitis e virus has been isolated (135). The other candidate vaccine has been evaluated in phase 2 and 3 clinical trials. It proved safe and efficacious against infection in seronegative participants in a phase 2 trial (135). To test its efficacy among the general population, the phase 3 trial included all age groups (16 to 65 years) and pregnant women with or without antibodies against HEV, from a region where both genotypes 1 and 4 co-circulate with the zoonotic genotype 4 predominating. The efficacy of this 3-dose vaccine after 1 year post-vaccination was 100%. Additionally, vaccination also proved beneficial under less than perfect conditions, for example when participants did not receive all 3 doses. Vaccine efficacy after two doses was also 100%

(166). Side effects to both vaccines were few and mild, with no serious adverse effects related to vaccination (135, 166).

HEV infection can, therefore, be controlled with an efficient immunization program where individuals in endemic areas as well as naïve travelers from non-endemic regions could also be protected. In industrialized countries, populations at risks such as transplant recipients or people with underlying liver conditions could also be candidates for vaccination. Due to the subclinical nature of the disease in animals, coupled with the low economic impact and the cost of vaccination, it is unlikely that a HEV vaccine would be a priority for animal producers and veterinarians. If an animal vaccine against HEV were to be developed, however, it would likely be as effective as the human vaccine at preventing HEV infection and spread among populations of animals, and in turn would prevent zoonotic transmission to humans.

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Chapter 2: Identification and Complete Genomic Sequence of the First Strain of Hepatitis E Virus (HEV) from Rabbits in the United States

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Emerg Infect Dis. 2011 Nov;17(11):2047-9.

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J. Virol. 2012 Dec;86(23):13124-5.

ABSTRACT

We report genetic identification of the first strains of hepatitis E virus (HEV) from rabbits in the United States. HEV RNA was detected in 14/85(16%) rabbit serum and 13/85(15%) fecal samples, and IgG anti-HEV in 31/85(36%) sera. After the complete genome sequence of the virus was determined, sequence and phylogenetic analyses indicated that the U.S. rabbit HEV is a distant member of the zoonotic genotype 3 HEV, thus raising a potential concern for zoonotic human infection. A unique 90 nt insertion within the X domain of the ORF1 was identified in the rabbit HEV and it is likely that this insertion may play a role in the species tropism of HEV.

Keywords: hepatitis E, hepatitis E virus, viruses, rabbits, genotype 3, zoonoses, zoonotic infection, animal reservoirs, Virginia, United States

INTRODUCTION

Hepatitis E virus (HEV) is an important human pathogen in the family of *Hepeviridae* (22). Hepatitis E is an acute self-limiting disease with a mortality of less than 1% in the general population, however the mortality can reach up to 25% in infected pregnant women (1, 9, 28). Chronic HEV infection with considerable morbidity and mortality has recently been documented in immunocompromised individuals such as organ transplant recipients (6, 16, 18).

As a zoonotic pathogen, HEV has been genetically identified from humans and a number of other animal species including chicken, pig, deer, rabbit, cutthroat trout, mongoose, rat, bat, and ferret (2, 3, 5, 14, 15, 21, 25, 27, 29). The first animal strain of HEV, swine HEV, is zoonotic and infects humans (20, 21). Among the four recognized major genotypes of HEV, the genotypes 1 & 2 are restricted to humans, whereas genotypes 3 & 4 are zoonotic and known to infect several other animal species (4, 10, 17, 22, 24). A unique strain of HEV was recently identified from farmed rabbits in China (7, 29), although its prevalence in other regions of the world remains unknown.

The objectives of this study were to determine if farmed rabbits in the United States are infected by HEV, and if so, to genetically identify and characterize the first strains of HEV from rabbits in the United States.

MATERIALS AND METHODS

Sample Collection from Farmed Rabbits in Virginia

Fecal swabs and serum samples were collected from a total of 85 rabbits from two rabbitries in Virginia (25 rabbits from Farm A, and 60 rabbits from Farm B). The two rabbitries were located in different regions of Virginia, however they both raised rabbits for human

consumption as well as for pets and fur. The ages of the rabbits on each farm varied greatly. On farm A there was an age range of 3.9 to 36.8 months with an average age of 7.0 months, while on farm B there was an age range of 3.0 to 56.9 months with an average age of 10.8 months. The rabbits were of various breeds including Californian, Flemish X, Lop, MiniRex, New Zealand, New Zealand X, Rex X, Salitan, and TN Redback.

Detection of HEV Antibodies by ELISA

Serum samples were tested for IgG anti-HEV with an ELISA assay essentially as described (24). A truncated recombinant HEV capsid protein containing the immunodominant region 452-617 aa (GenWay Biotech Inc) was used as antigen. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (KPL) was used as the secondary antibody. Preimmune and immune anti-HEV rabbit sera were included as negative and positive controls, respectively. Anti-HEV immune serum was obtained from two rabbits intravenously inoculated with an HEV rabbit strain isolated previously from China. Preimmune serum was collected from the two rabbits prior to inoculation. The ELISA cutoff was calculated as the mean negative control optical density (O/D) value plus three standard deviations.

Detection of Rabbit HEV RNA by RT-PCR

All rabbit serum and fecal swab samples were tested for the presence of HEV RNA by a nested RT-PCR assay using a set of degenerate primers that amplify a conserved region in the HEV capsid gene. RNA extraction was performed using a standard Trizol Reagent protocol on all rabbit serum and fecal samples. Briefly, 200 μ l of serum and 200 μ l of a 10% fecal suspension were used for the RNA extraction. The total RNA was resuspended in 30 μ l of DNase, RNase, and proteinase-free water (Invitrogen). Reverse transcription was performed on 12.25 μ l of each RNA sample for 60 min at 42°C using 1 μ l of the reverse primer, 0.25 μ l

(20u/μl) Superscript II reverse transcriptase (Invitrogen), 4 μl of 5 x RT Buffer, 1 μl of 0.1 M dithiothreitol, 0.50 μl (40u/μl) RNase inhibitor (Promega), and 1μl of 10mM deoxynucleoside triphosphates. Five microliters of the resulting cDNA was then amplified in a 50 μl PCR reaction with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The PCR parameters used in this study are essentially the same as described previously (11).

The degenerate primers were designed based on a multiple sequence alignment of the two known Chinese rabbit strains of HEV (8, 29) as well as 75 other genotype 3 HEV strains available from the GenBank database: first round PCR, forward primer RabdegF1 5'-GCMACACGKTTYATGAARGA-3', reverse primer RabdegR1 5'-ACYTTRGACCAATCVAGRGARC-3'; second round PCR, forward primer RabdegF2 5'-GCTGAYACRCTTCTYGGYG-3', reverse primer RabdegR2 5'-TGAMGGRGTRGGCYGRTCYTG-3'.

Determination of Complete Genome Sequence of first strain of U.S. Rabbit HEV and Sequence Analyses

Using a HEV-positive fecal sample collected from a rabbit farm in Virginia, overlapping fragments covering the complete genome of U.S. rabbit HEV were subsequently amplified using the primer walking strategy. For this technique, forward and reverse primers for each overlapping fragment were designed by incorporating the known sequences of the extreme 3' or 5' ends, respectively, of the sequenced portion of the U.S rabbit HEV genome. Degenerate primers corresponding to the other unknown 5' or 3' end of each known primer set were designed by aligning the existing strains of rabbit HEV from China with the known fragment of the U.S. strain. The RNA extraction and nested RT-PCR protocol used to amplify each of the overlapping fragments were essentially the same as described in the previous section.

The extreme 5' and 3' ends of the viral genome were amplified according to manufacturer's instructions using the 5' and 3' RACE System for Rapid Amplification of cDNA Ends (Invitrogen). RACE is a procedure for amplification of nucleic acid sequences from mRNA templates between a defined internal site and unknown sequences at either the 3' or 5' end of the template.

The complete genomic sequence was assembled and analyzed using the MegAlign computer software (DNASTAR, Inc., Madison, WI).

RESULTS

Prevalence of IgG HEV Antibodies

The prevalence of IgG anti-HEV in the rabbits in Virginia was 36.5% (31/85), with 52% (13/25) for Farm A rabbits and 30% (18/60) for farm B rabbits (Table 1).

Detection of Novel Strains of HEV from Farmed Rabbits in the United States

HEV RNA was detected in 19/85 (22%) rabbits, including 14/85 serum (16%) and 13/85 (15%) fecal samples (**Table 1**). The authenticity of the amplified PCR products was confirmed by sequencing. More rabbits were positive for HEV RNA in farm A (48% and 40% in serum and fecal samples, respectively) than in farm B (3% and 5% in serum and fecal samples, respectively) (**Table 1**). The total numbers of rabbits from the two farms that were infected (fecal shedding, viremic or seropositive) were 42/85 (49%) for both farms, with 20/25 (80%) in farm A and 22/60 (37%) in farm B (**Table 1**).

Sequence and Phylogenetic Analyses Demonstrated that the Novel U.S. Rabbit HEV Strains Belong to Genotype 3

A 181-bp sequence within the capsid gene was determined for all 27 PCR-positive samples amplified from the rabbits. Sequence analyses identified a total of 4 different HEV isolates: USRab-14, USRab-16, USRab-31, and USRab-52. The 4 rabbit HEV isolates shared approximately 81.2-97.8% nucleotide sequence identity with each other, and 80.1-95.6% nucleotide sequence identity with the two Chinese rabbit HEV isolates GDC9 and GDC46 (7, 29). The small amounts of clinical samples (fecal swabs and sera) collected from the rabbits limited our ability to perform extensive genetic characterization of the rabbit HEV isolates. However, we did obtain a larger 765-bp sequence within the capsid gene of isolate USRab-14 (**Fig. 1**). A set of hemi-nested primers was used to amplify this fragment; first round of PCR was performed using RabdegF2 and RabOrf2R1 (reverse, 5'-TTAAAACTCCCGGGTTTTACC-3'). A second round of PCR was performed using RabOrf2F2 (forward, 5'-CAGGTATTCTACTCCCGC-3') and RabOrf2R1.

Sequences analysis based on the 765-bp sequence revealed that the USRab-14 isolate shared approximately 87-89% nucleotide sequence identity with the two Chinese rabbit strains of HEV (**Table 2**), and phylogenetic analysis revealed that the USRab-14 isolate grouped together with the two Chinese rabbit strains (GDC9 and GDC46) (**Fig. 1**). The rabbit strains of HEV are more closely related to the genotype 3 HEV than to any other known HEV genotypes (**Fig. 1, Table 2**), suggesting that the rabbit HEV is likely a distant member of genotype 3 (24).

Complete Genome Sequence and Sequence Analyses of first strain of U.S. Rabbit HEV

Excluding the poly(A) sequence, the complete genome of the U.S. rabbit HEV is 7,282 bp in length and the G/C content is 55.6%. The genome organization of the U.S. rabbit HEV is similar to other mammalian HEVs with a 5' untranslated region (UTR) (nt 1-26), followed by ORF1 (nt 27-5195), ORF2 (nt 5230-7212), ORF3 (nt 5192-5560), and the 3' UTR (nt 7213-

7,306). The complete genome sequence of the first U.S. rabbit HEV strain was deposited in the GenBank database under accession number JX565469. Compared to the known genotypes 1-4 mammalian HEVs, a unique 90 nt insertion within the X domain of the ORF1 was identified in the rabbit HEV. The U.S. rabbit HEV shares approximately 74%, 73%, 79% and 75% nucleotide sequence identities across the entire genome with the genotypes 1, 2, 3 and 4 mammalian HEVs, respectively. Phylogenetic analysis revealed that the rabbit HEV is a distant member of the genotype 3 HEV.

DISCUSSION

This study reports for the first time that farmed rabbits in the United States are naturally infected by HEV. IgG anti-HEV and HEV RNA were detected in various breeds of rabbits from two different farms in Virginia. The prevalence of IgG anti-HEV and HEV RNA varied between the two rabbitries, being significantly higher in farm A than in farm B. This variation may reflect the difference in rabbit housing practice in the two farms: rabbits in farm A were caged in groups of 2-9 whereas rabbits in farm B were all caged individually. Since HEV is transmitted fecal-orally, the virus likely spreads between cage mates in farm A, and thus increasing the numbers of HEV-positive rabbits.

The overall prevalence of IgG anti-HEV (36%) in the U.S. rabbits tested was lower than that in rabbits from Gansu and Beijing, China (57% and 55%, respectively) (7, 29), whereas the prevalence of HEV RNA in serum and fecal samples from the U.S. rabbit farms (16.5% and 15.3% respectively) was higher than that in Gansu and Beijing, China (7.5% and 6.96% respectively). Again, the ages of rabbits tested, the animal housing practice, and hygienic conditions of the rabbitries may explain the observed difference in HEV RNA and antibody prevalence.

The U.S. rabbit HEV belongs to genotype 3 HEV, which is zoonotic and capable of infecting across species barriers (17, 18, 23, 26). The U.S. rabbit HEV contains a unique 90 nt insertion within the X domain of the ORF1 (13). Since a 171-nucleotide insertion of human S17 ribosomal protein gene in the hypervariable region adjacent to the X domain of a genotype 3 HEV genome has been linked to an expanded host range of cross-species infection in cultured cells (26), this unique 90-nt insertion in rabbit HEV may play a role in species tropism.

It appears that, like swine HEV in pigs (21) and avian HEV in chickens (12), rabbit HEV is also widespread in the rabbit population in the United States. Future studies are warranted to comprehensively assess the prevalence of HEV in rabbits from different geographic regions. The fact that the rabbit HEV is closely related to genotype 3 HEV raises a concern for potential zoonotic infection of humans, especially rabbit farmers, since genotype 3 HEV from other animal species such as swine and deer is known to infect humans (18, 19). Therefore, it will be important to evaluate the ability of cross-species infection and zoonotic risk of rabbit HEV in the future.

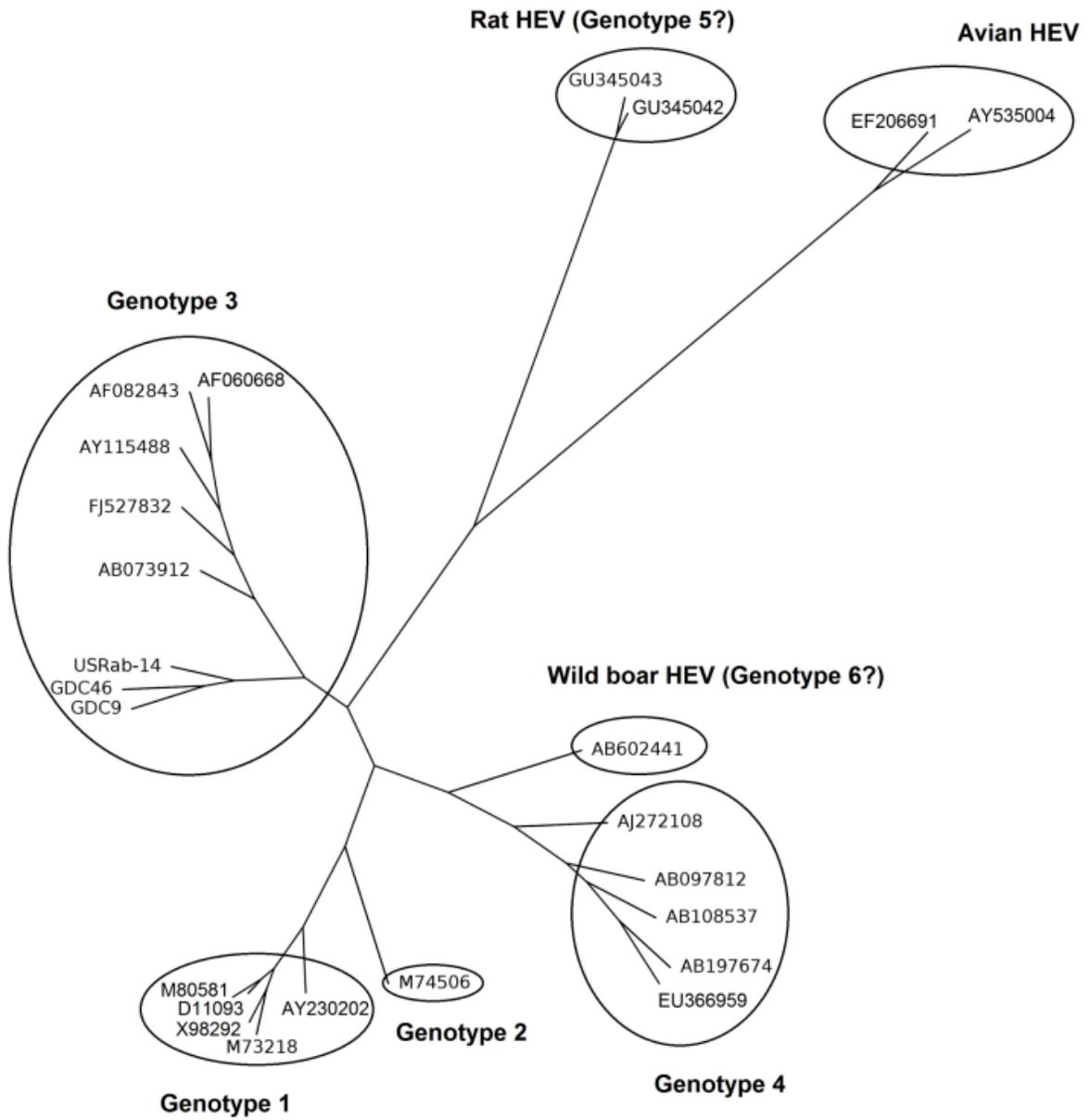
ACKNOWLEDGEMENTS

We thank the rabbitry farmers for allowing us to collect rabbit samples, Lynn Chipkin for helping with sample collection, and Nathan Beach for assistance with phylogenetic analysis. This study was supported in part by grants (R01AI050611, and R01AI074667) from the U.S. National Institutes of Health.

FIGURES

Figure 1. A phylogenetic tree based on the 765-bp sequence of the ORF2 capsid gene of the U.S. rabbit HEV isolate USRab-14, two Chinese rabbit HEV isolates (GDC9 and GDC46), representative genotypes 1-4 HEV strains, avian HEV, rat HEV, and a novel boar HEV. The GenBank accession numbers are used to identify each HEV strain used in the phylogenetic analysis.

Figure 1.



TABLES

Table 1: Detection of HEV antibody in sera and HEV RNA in serum and fecal samples of rabbits from two rabbitries in Virginia.

Farm	No. of rabbits tested	Mean age (months)	No. of positive for IgG anti-HEV (%)	No. of positive for HEV RNA (%)		Total no. of rabbits exposed to HEV (%)
				Serum	Feces	
A	25	7.0	13 (52.0)	12 (48.0)	10 (40.0)	20 (80.0)
B	60	10.8	18 (30.0)	2 (3.3)	3 (5.0)	22 (36.7)
Total	85	9.7	31 (36.5)	14 (16.5)	13 (15.3)	42 (49.4)

Table 2: Percentage of nucleotide sequence identities of a 765-bp capsid gene sequence among U.S. and Chinese rabbit strains of HEV, genotype 1-4 HEV, and avian HEV.

	Chinese Rabbit HEV	Genotype 1 HEV	Genotype 2 HEV	Genotype 3 HEV	Genotype 4 HEV	Avian HEV
U.S. rabbit HEV (USRab-14)	87.2-89.0	78.3-79.3	74.8	80.1-82.3	78.9-81.0	34.9-35.9
Chinese Rabbit HEV		77.2-78.6	74.5-75.5	78.7-83.1	78.0-82.0	35.3-36

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**Chapter 3: Cross-species infection of pigs with a novel rabbit, but not rat,
strain of hepatitis E virus isolated in the United States**

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J Gen Virol. 2012 Aug;93(Pt 8):1687-95.

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ABSTRACT

Hepatitis E virus (HEV) is an important human pathogen. In addition to humans, HEV has also been identified in pig, chicken, mongoose, deer, rat, rabbit and fish. There are four recognized and two putative genotypes of mammalian HEV. Genotypes 1 and 2 are restricted to humans, while genotypes 3 and 4 are zoonotic. The recently identified rabbit HEV is a distant member of genotype 3. Here we first expressed and purified the recombinant capsid protein of rabbit HEV, and showed the capsid protein of rabbit HEV cross-reacted with antibodies raised against avian, rat, swine and human HEV. Conversely, we showed that antibodies against rabbit HEV cross-reacted with capsid proteins derived from chicken, rat, swine and human HEV. Since pigs are the natural host of genotype 3 HEV, we then determined if the rabbit HEV infects pigs. Twenty pigs were divided into 5 groups of 4 each, and intravenously inoculated with PBS, U.S. rabbit HEV, Chinese rabbit HEV, U.S. rat HEV, and swine HEV, respectively. Results showed that only half of the pigs inoculated with rabbit HEV had low levels of viremia and fecal virus shedding, indicative of active but not robust HEV infection. Infection of pigs by rabbit HEV was further verified by transmission of the virus recovered from pig feces to naïve rabbits. Pigs inoculated with rat HEV showed no evidence of infection. Preliminary results suggest that rabbit HEV is antigenically related to other HEV strains and infects pigs, and that rat HEV failed to infect pigs.

Keywords: Hepatitis E Virus (HEV), cross-species, transmission, zoonosis

INTRODUCTION

Hepatitis E virus (HEV), the causative agent of hepatitis E, is an important human pathogen. Sporadic cases of acute hepatitis E have also been reported in many industrialized countries including the United States (26, 49). HEV is transmitted primarily by the fecal-oral route and causes self-limiting acute hepatitis with a high morbidity in young adults (10, 11, 22, 25, 26). At least four major genotypes of HEV have been recognized thus far (2, 23, 24, 37): genotypes 1 and 2 are restricted to humans, whereas genotypes 3 and 4 have an expanded host range and are zoonotic (3, 6, 14, 24, 25, 32). Recently, two potential new genotypes of HEV were identified from rats in Germany and the United States (20, 36) and from wild boars in Japan (40, 45). Avian HEV from chickens likely represents a new genus within the family *Hepeviridae* (5, 25, 26). The strain of HEV recently identified from cutthroat trout appears to belong to a new genus as well (4).

HEV is a small, non-enveloped virus with a positive-sense RNA genome of approximately 7.2kb (1, 32). The virus contains three open reading frames (ORF): ORF1 encodes non-structural proteins, ORF2 encodes the viral capsid protein, and ORF3 encodes a cytoskeleton-associated phosphoprotein with multiple functions (19, 25, 43, 48, 50). The recent availability of cell culture systems for HEV will aid future studies of HEV biology (31, 33). The first isolation of a non-human animal strain of HEV was from a pig in the United States in 1997, designated swine HEV (29), and since then swine HEV has been genetically identified from pigs worldwide (24). Thus far, all strains of HEV identified from pigs belong to either genotype 3 or 4, although a putative new genotype was recently identified from a wild boar (45). Genotypes 3 and 4 strains of HEV can infect across species barriers and are zoonotic (24, 25, 34, 41). In addition to pigs and humans, genetically divergent strains of HEV have also been isolated from

several other animal species including chicken, rat, mongoose, horse, deer, and rabbit (17, 20, 22, 28, 30, 38, 42, 44, 51).

The novel rabbit strain of HEV identified from farmed rabbits in China is distantly related to the genotype 3 (13, 51). More recently, we genetically identified a novel strain of HEV from rabbits in the United States (9) that is also distantly related to the genotype 3. We showed that infection of rabbits by genotype 3 HEV is potentially widespread in the United States (9). It appears that the rabbit strains of HEV from both China and the United States belong to the genotype 3 (9, 37).

A genetically distinct strain of HEV, designated rat HEV, was identified from Norway rats in Germany and shares approximately 60% and 50% nucleotide sequence identity with human and avian strains of HEV, respectively (20). More recently, a rat HEV similar to that from Germany was identified from urban rats in Los Angeles, California (36). Transmission of the U.S. rat HEV to naïve laboratory rats was successful, although the transmission was spotty and did not result in a robust infection. An attempt to transmit the U.S. rat HEV to rhesus monkeys was unsuccessful, suggesting that rat HEV is likely not a source of HEV infection in humans (36).

Since pigs are the natural host of the genotype 3 HEV, it is possible that, like swine HEV (28), the rabbit strain of HEV may also have the ability to infect across species. Therefore, the objectives of this study were to determine whether the rabbit and rat strains of HEV can cross species barriers and infect pigs, and whether there is an antigenic cross-reactivity in the capsid protein between the rabbit strain of HEV and other known animal strains of HEV.

MATERIALS AND METHODS

Sources of Viruses

The United States strain of rabbit HEV (USRab14) was a 10% fecal suspension in PBS buffer (w/v) prepared from feces of farmed rabbits in Virginia that are positive by PCR for HEV RNA (9). The Chinese strain of rabbit HEV was from a serum sample of rabbits that were experimentally infected with the RC-39 strain of rabbit HEV isolated in China (22, 51). The U.S. rat strain of HEV was a homogenate of liver from a laboratory rat experimentally infected with the U.S. strain of rat HEV with an infectious dose titer of $10^{5.9}$ per 0.5 ml (36). The genotype 3 swine HEV used as the positive control was from an experimentally infected pig with a titer of $10^{4.5}$ 50% pig infectious dose (PID₅₀) (12, 15, 28, 39).

Animals

Twenty, 6-week old, cross-bred specific-pathogen-free (SPF) pigs were obtained from the Virginia-Maryland Regional College of Veterinary Medicine's Swine Breeding Facility for the cross-species transmission study. Prior to inoculation, all pigs were confirmed negative for IgG anti-HEV by an enzyme-linked immunosorbent assay (ELISA). Six, 8-week old, New Zealand White rabbits were obtained from Harlan Laboratories for the rabbit infection study. Prior to inoculation, each rabbit was confirmed negative for IgG anti-HEV by an ELISA.

Generation of an infectious stock of the Chinese rabbit strain of HEV

In order to generate an infectious virus stock of the Chinese rabbit HEV, a serum sample positive by RT-PCR for a Chinese rabbit HEV (RC-39) was used to inoculate two naïve rabbits (#560 and #562). Briefly, the rabbits were intravenously inoculated with 0.5 ml of a RT-PCR-positive serum. Feces were collected every other day, and blood was collected once a week from each rabbit. Rabbit #560 was necropsied during the acute stage of infection at 22 days post-infection (dpi). Intestinal content and bile collected during the necropsy were prepared in a 10%

suspension (w/v in PBS) and used as an infectious stock of the Chinese rabbit HEV. Rabbit #562 was kept until 56 dpi to determine seroconversion over time.

Experimental inoculation of pigs with rabbit and rat strains of HEV

The 20 SPF pigs were divided into 5 groups of 4 pigs per group. Each group was housed in a separate room within a climate-controlled Biosafety Level 2 (BSL-2) facility and had access *ad libitum* to food and water. Each group of pigs were inoculated intravenously with phosphate-buffered saline (PBS) as a negative control, U.S. strain of rabbit HEV, Chinese strain of rabbit HEV, a U.S. strain of rat HEV, and a genotype 3 swine HEV as a positive control, respectively. A strict biosecurity protocol was followed for feeding and sample collection. Serum and fecal samples were collected weekly from each pig at 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70 dpi. Serum samples were tested for respective viral RNA (viremia) by RT-PCR essentially as described (7, 8, 18) and for anti-HEV IgG by ELISA as previously described (29). Fecal samples (10% fecal suspension in PBS) were also tested for respective HEV RNA by RT-PCR. All pigs were necropsied at 10 weeks post-inoculation. At necropsy, samples of serum, intestinal content, bile, and liver tissue were collected and stored at -80°C until use.

Experimental inoculation of rabbits with rabbit HEV recovered from experimentally infected pigs and from farm rabbits

To further confirm that pigs experimentally inoculated with the rabbit HEV are indeed infected and excrete infectious virus in feces, we subsequently inoculated rabbits with a suspension of a RT-PCR-positive feces collected from a pig experimentally inoculated with the rabbit HEV. Rabbits were housed in separate cages within a climate-controlled BSL-2 facility and had access *ad libitum* to water and a controlled amount of food throughout the study. Two

rabbits (ID #1, and #3) were each inoculated with a 10% suspension of a RT-PCR-positive feces collected from pigs experimentally inoculated with the U.S. rabbit strain of HEV (USRab-14). Two other rabbits (ID #7, and #8) were each inoculated with a 10% fecal suspension containing the USRab-14 prepared from feces directly collected from farmed rabbits in Virginia (9). Fecal samples were collected every other day from each rabbit following inoculation. Serum samples were collected at 0, 14, 21, 28, 35, 42, 49, 56, 63 dpi. Serum samples were tested for rabbit HEV RNA by RT-PCR essentially as described (7, 8, 18) and for anti-HEV IgG by ELISA as previously described (29). Fecal samples (10% fecal suspension in PBS) were also tested for rabbit HEV RNA by RT-PCR. At each necropsy, samples of serum, intestinal content, bile, and liver tissue were collected and stored at -80C until use.

Detection and semi-quantitative titration of HEV RNA in samples by nested RT-PCR

RNA extraction was performed using a standard Trizol Reagent protocol on all pig and rabbit serum and fecal samples. Briefly, 200 µl of serum and 200 µl of a 10% fecal suspension were used for the RNA extraction. Additionally, total RNAs were also extracted from 200 µl of bile, 10% (w/v) intestinal content in PBS, and 10% suspension of liver homogenates. Reverse transcription was performed on 12.25 µl of each RNA sample for 60 min at 42°C using 1µl of the virus strain-specific reverse primer (Table 1), 0.25 µl (20u/µl) Superscript II reverse transcriptase (Invitrogen), 4 µl of 5 x RT Buffer, 1 µl of 0.1 M dithiothreitol, 0.50 µl (40u/µl) RNase inhibitor (Promega), and 1µl of 10mM deoxynucleoside triphosphates. Five microliters of the resulting cDNA was then amplified in a 50 µl PCR reaction with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The PCR parameters used in this study are essentially the same as described previously (18) using virus strain-specific primers that amplify a capsid gene region.

The strain-specific PCR primers for the RT-PCR assays to detect respective HEV RNA in pigs experimentally inoculated with U.S. rabbit strain, Chinese rabbit strain, rat strain, and the genotype 3 swine HEV are listed in Table 1. The strain-specific primers were designed based on the published sequences of U.S. rabbit HEV (9), Chinese rabbit HEV (51), U.S. rat HEV (36), and genotype 3 swine HEV (29). In addition, degenerate primers that can amplify known genotype 3 strains of HEV were also designed based on a multiple sequence alignment of the two known Chinese rabbit strains of HEV (13, 51) as well as 75 other genotype 3 HEV strains.

To further confirm the RT-PCR results, all serum and fecal samples that were tested positive in pigs experimentally inoculated with U.S. rabbit HEV were subsequently retested with a different nested RT-PCR assay using primers that amplify a different genomic region in ORF1. The rabbit serum and fecal samples were similarly tested with USRabF1, USRabF2, USRabR1, and USRabR2 primers (Table 1).

Additionally, the amount of HEV RNA present in the fecal samples that had tested positive by the nested RT-PCR was estimated using a semi-quantitative nested RT-PCR assay and calculated as genome equivalent (GE) per mL of the sample (21, 27, 28). One GE is defined as the number of viral genomes present in the highest 10-fold dilution that is tested positive by RT-PCR. The same species-specific primers described above were used for the semi-quantitative nested RT-PCR assay (Table 1).

ELISA to detect IgG anti-HEV in pigs and rabbits

Serum samples from experimentally-inoculated pigs were tested for IgG anti-HEV using an ELISA essentially as described (29). A truncated recombinant genotype 1 HEV capsid protein containing the immuno-dominant 452-617aa region (GenWay Biotech, Inc) was used as the antigen. Horseradish peroxidase (HRP)-conjugated goat anti-swine IgG (KPL) was used as the

secondary antibody. Preimmune and convalescent-phase sera from pigs that were experimentally infected with genotype 3 swine HEV (8), and a hyper-immune pig antiserum from a pig that was immunized with a recombinant capsid protein of the rat HEV (Sanford et al., unpublished data) were included as negative and positive controls for pigs inoculated with rabbit HEV and rat HEV, respectively.

To further confirm the serology results in pig sera obtained with the ELISA using the genotype 1 HEV antigen described above, we subsequently performed a separate ELISA using species-specific rat HEV and rabbit HEV antigens on all sera from pigs inoculated with rat HEV and rabbit HEV, respectively. A truncated recombinant rat HEV capsid protein (100-660 aa region) and a truncated recombinant rabbit HEV capsid protein (390-660 aa region) were used as the species-specific HEV antigens and the ELISA protocol was essentially the same as described (29).

Sera collected from rabbits were also tested for the presence of IgG anti-HEV by ELISA using HRP-conjugated goat anti-rabbit IgG as the secondary antibody. Pre-immune and convalescent-phase serum samples obtained from a rabbit experimentally infected with rabbit HEV were included as negative and positive controls, respectively. The ELISA cutoff for both assays was calculated as the mean negative control optical density (O/D) value plus three standard deviations.

Western blot analyses to determine antigenic cross-reactivity between the rabbit HEV and other known strains of HEV

To determine if rabbit HEV is antigenically related to other known animal strains of HEV, western blot analyses were performed with recombinant capsid antigens derived from different HEV strains and anti-HEV antibodies raised against different strains of HEV. First, to

determine if the recombinant capsid proteins derived from different strains of HEV cross-react with rabbit HEV antiserum (Figure 2A), each lane of a 8-16% SDS-PAGE gel was loaded with the same amount (1 μ g) of recombinant capsid proteins derived from different HEV strains including the U.S. rabbit HEV (34kDa), the genotype 1 human HEV (43 kDa), the genotype 3 swine HEV (60kDa), avian HEV (32kDa) (16), and rat HEV (60kDa) (Sanford et al., unpublished data). The size variation of these recombinant capsid proteins reflects the size difference of the capsid gene from different HEV strains as well as different sizes of truncation. After separation of the proteins in the gel, the proteins were stained with Bio-Safe Coomassie Stain (Bio-Rad) for Coomassie-staining analysis. The separated protein was transferred to a polyvinylidene difluoride (PVDF) membrane, which was subsequently blocked for 1 hr at room temperature with Odyssey blocking buffer (Licor Biosciences). The membrane was then cut into two separate pieces, the first membrane containing truncated capsid proteins from rabbit HEV, genotype 1 HEV, genotype 3 HEV, avian HEV and rat HEV was incubated overnight with 1:100 dilution of a rabbit HEV antiserum (3 ml Odyssey blocking buffer, 30 μ l antiserum, 3 μ l Tween-20). The second membrane, containing the truncated capsid protein derived from the rabbit HEV, was incubated with 1:100 dilution of a rabbit serum known to be negative for HEV antibodies as a negative control. Following incubation with the primary antibodies, the membrane pieces were washed in washing buffer (0.2% Tween-20 PBS Solution) and then incubated with 1:5000 dilution of Infrared IRDye 680LT goat anti-rabbit secondary antibody (LI-COR) for 1 hr. After washing 3 times with the washing buffer, the membrane pieces were scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR) in the 700 nm channel.

Secondly, to determine if the recombinant rabbit HEV capsid antigen cross-reacts with antibodies raised against different HEV strains (Figure 2C), each lane of a SDS-PAGE gel was

loaded with the same amount (1 μ g) of the recombinant truncated capsid protein derived from the U.S. rabbit HEV (USRab-14). After transferring the separated protein to a PVDF membrane, the membrane was subsequently blocked for 1 hr in Odyssey blocking buffer. The membrane was then cut into separate pieces with each containing one lane, and each membrane piece was separately incubated with 1:100 dilution of respective primary anti-HEV antiserum against different strains of HEV including a rabbit HEV antiserum from a rabbit experimentally-infected with U.S. rabbit HEV as the positive control, a genotype 1 human HEV hyper-immune antiserum from a pig immunized with the capsid protein of genotype 1 human HEV (29), a genotype 3 HEV antiserum from a pig experimentally-infected with a genotype 3 swine HEV (8), an avian HEV antiserum from a chicken experimentally-infected with avian HEV (35), a rat HEV antiserum from a pig immunized with a recombinant truncated capsid protein of rat HEV (Sanford *et al.*, unpublished data), and a pre-immune rabbit serum as the negative control. Following incubation with each of the primary antibodies, the membrane pieces were washed and then incubated for 1 hr with 1:5000 dilution of the respective infrared secondary antibody. The secondary antibodies were infrared IRDye 680LT goat anti-rabbit, goat anti-swine, and goat anti-chicken (LI-COR) depending on the host species of the primary antiserum used. The membrane pieces were subsequently washed 3 times, and then scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR) in the 700 nm channel.

An ELISA was used to estimate the antibody titers of the different HEV antisera used for the western blot analyses essentially as described above (29) using the truncated recombinant genotype 1 HEV capsid protein (GenWay Biotech, Inc) as the antigen since it has been shown to cross-react with all of the strains of HEV tested in the study. Ten-fold serial dilutions (1:10, 1:10², 1:10³, 1:10⁴, 1:10⁵) of each antiserum were performed. Species-specific horseradish

peroxidase (HRP)-conjugated goat anti-swine IgG, goat anti-rabbit IgG, or goat anti-chicken IgG were used as the secondary antibody depending on the source of the antiserum. Preimmune swine and rabbit sera were included as a negative control (data not shown).

RESULTS

Generation of infectious stocks of the Chinese and the U.S. strains of rabbit HEV

The two rabbits (#560 and #562) inoculated with the PCR-positive serum sample containing a Chinese strain of rabbit HEV became infected. Fecal shedding of the virus was detected at 10 days post-infection (dpi). At 22 dpi, rabbit #560, which had shed virus in feces for two consecutive weeks (Table 2), was euthanized. An infectious stock of Chinese rabbit HEV (strain RC39) was then prepared as a 10% suspension (w/v in PBS) of the intestinal content and bile collected during the necropsy of rabbit #560, and this infectious virus stock has a titer of approximately 2×10^6 genome equivalent (GE) per ml. The U.S. strain of rabbit HEV (USRab14) infectious virus stock was prepared as a 10% suspension of feces (w/v in PBS) collected from PCR-positive farmed rabbits in Virginia with an approximate titer of 2×10^5 GE per gram of feces. The semi-quantification of virus GE titers was essentially done as previously described (21, 46, 47).

Cross-species infections of specific-pathogen-free (SPF) pigs by U.S. and Chinese strains of rabbit HEV but not by the U.S. strain of rat HEV

Groups of 4 pigs were each inoculated with the U.S. rabbit HEV, Chinese rabbit HEV, U.S. rat HEV, genotype 3 swine HEV (positive control), and PBS buffer (negative control). Two pigs, one in the negative control group and one in the rat HEV-inoculated group, died prior to the termination of the project from causes unrelated to the infection. There was no detectable fecal

virus shedding in the negative control group. In pigs inoculated with the U.S. rabbit HEV, fecal virus shedding was sporadic and began at 5 week post-inoculation (wpi) in only 1/4 inoculated pigs, even though transient viremia was detected in 2/4 pigs. In pigs inoculated with the Chinese rabbit HEV, fecal virus shedding was apparent in 2/4 pigs beginning at 6 wpi (Table 3.). In pigs inoculated with a genotype 3 swine HEV, fecal virus shedding began as early as 1 wpi, and 4/4 positive control pigs shed virus in feces, and 3/4 pigs were viremic starting at 2 wpi (Table 3.). In pigs inoculated with the U.S. rat HEV, there was no detectable fecal virus shedding or viremia (Table 3). Seroconversion to IgG anti-HEV was detected in pigs inoculated with swine HEV but not in pigs from any other groups using genotype 1 human HEV, rat HEV or rabbit HEV antigens (data not shown).

All liver and bile samples collected during necropsies at 10 wpi from pigs in the negative control group and in groups inoculated with U.S. rabbit HEV and rat HEV were tested negative for HEV RNA. In the group inoculated with Chinese rabbit HEV, 2 pigs were positive for HEV RNA in the bile, and one also positive for HEV RNA in the liver. All 4 pigs in the positive control group were positive for HEV RNA in the liver, but none in bile (Table 3).

A semi-quantitative nested RT-PCR was performed on the positive fecal samples from pigs that had been inoculated with U.S. rabbit HEV and Chinese rabbit HEV. The approximate GE titer of the positive fecal samples from pigs infected by the U.S. rabbit HEV ranged from 10^3 to 10^5 GE per gram of feces, while the approximate GE titer of the fecal samples from pigs infected by the Chinese rabbit HEV ranged from 10^2 to 10^6 GE per gram of feces, indicating a low level of replication of the rabbit HEV in pigs.

The virus recovered from feces of pigs experimentally infected with rabbit HEV can infect naïve rabbits under laboratory conditions

To further confirm if pigs are indeed infected by rabbit HEV and excrete infectious virus in feces, two rabbits (ID#1 and #3) were intravenously inoculated with a 10% suspension (w/v in PBS) of feces collected from pigs inoculated with the rabbit HEV USRab-14 that were tested positive for HEV RNA by RT-PCR. The two inoculated rabbits begin to shed virus sporadically in feces at 12 dpi and the fecal virus shedding continued until the 6th wpi (Table 2). In addition, as a positive control, we also inoculated two other rabbits (ID#7 and #8) with a 10% suspension of feces prepared from farmed rabbits in Virginia that were tested positive by RT-PCR for rabbit HEV RNA. These two inoculated rabbits began to shed virus in the feces at 6 dpi and continued to be positive until euthanasia (Table 2). One of these two rabbits, ID#8, was euthanized during the acute stage of infection (third week of the study) in order to prepare a higher titer virus stock of USRab-14 for future studies.

Rabbits ID#7 and #8 also seroconverted to IgG anti-HEV (Fig. 1), although rabbit #8 was euthanized at third week post-inoculation. However, the two other rabbits (ID#1 and #3) had no detectable seroconversion (Fig.1). The bile, intestinal content and liver collected during necropsies from rabbits #7 and #8 were tested positive for rabbit HEV RNA. Similarly, the liver and intestinal content from rabbit #1, but not #3, were also tested positive for rabbit HEV RNA.

Rabbit HEV is antigenically related to avian, rat, swine, and human HEVs

To determine antigenic cross-reactivity, we performed Western blot analyses. The results showed that the rabbit HEV antiserum cross-reacted with truncated recombinant capsid antigens derived from avian, rat, swine and human HEV strains (Fig. 2A). Reactivity was demonstrated between the purified truncated capsid protein of rabbit HEV and rabbit HEV antiserum (Fig. 2A). The truncated genotype 1 human HEV capsid protein (GenWay) and the truncated genotype

3 swine HEV capsid protein also demonstrated overt reactivity with the rabbit HEV antiserum, whereas the cross-reactivity between the truncated avian HEV (16) and rat HEV capsid proteins with the rabbit HEV antiserum was weaker, but still evident. The rabbit HEV recombinant capsid protein did not react with pre-immune serum from rabbits (Fig. 2A). The rabbit HEV capsid protein did not react with pre-immune serum from rabbits (Fig. 2A). The purity of the respective HEV antigens was demonstrated in the Coomassie-staining gel (Fig. 2B). The extraneous bands especially the lower molecular weight bands that appear in some lanes (Fig. 2A) may be due to protein degradation, which is not uncommon in western blots.

Similarly, a truncated purified recombinant capsid protein of rabbit HEV demonstrated some degree of cross-reactivity with the antisera raised against all of the different strains of HEV that were tested in this study (Fig. 3). However, quantification of the degree of antigenic cross-reactivity in this experiment is not possible since the antibody titer of each antiserum is unknown. To estimate the anti-HEV antibody level of these different HEV antisera used in the western blot analysis, we performed an ELISA on 10-fold serially dilutions of each antiserum. The genotype 3 HEV pig antiserum had the highest level of anti-HEV antibodies compared to the other HEV antisera (data not shown), thus explaining the observed high level of cross-reactivity between the genotype 3 HEV pig antiserum and the rabbit HEV antigen (Fig. 3).

DISCUSSION

Hepatitis E is a recognized zoonotic disease; pigs and likely other animal species are reservoirs for HEV (24-26). Recently, a novel genotype 3 strain of HEV was identified in rabbits first from China (51) and subsequently in the United States (9). More recently, another unique strain of HEV was identified in rats in Germany (20) and in the United States (36). Although the rat HEV could not be transmitted to rhesus macaques (36), the biological significance of the

rabbit HEV remains unclear. In this study, we aim to characterize the antigenic relatedness between the rabbit HEV and other known animal strains of HEV and to determine if the rabbit HEV and the U.S. rat HEV have the ability to infect across species.

As expected, the rabbit HEV (strain USRab-14) capsid antigen reacted with the rabbit HEV antiserum as well as with antisera against genotype 1 human HEV and genotype 3 swine HEV, suggesting that the rabbit HEV shares conserved antigenic epitopes with the genotypes 1 and 3 HEV. Interestingly, the rabbit HEV capsid antigen also reacted with antisera raised against the distantly-related avian HEV and rat HEV, further indicating that there exists only one serotype among mammalian HEV (10). The antigenic cross-reactivity was further verified in a reverse western blot analysis when the capsid antigens derived from genotypes 1 and 3 HEV were shown to react with rabbit HEV antiserum. Avian and rat HEV antigens also reacted with rabbit HEV antiserum. The results revealed that the rabbit HEV is antigenically related to the known mammalian HEV strains as well as the avian HEV strain.

Since the rabbit HEV is a distant member of the genotype 3 (37) and since pigs are the nature host of the genotype 3 HEV, we assessed the ability of rabbit HEV to cause cross-species infection in a pig model. We demonstrated that, indeed, both the U.S. strain and the Chinese strain of rabbit HEV were able to infect SPF pigs when inoculated intravenously as approximately half of the inoculated pigs developed transient viremia and shed the virus sporadically in feces. It appears that, when compared to the pigs infected with the genotype 3 swine HEV, the pigs infected with the rabbit HEV have a delayed onset and shorter duration of viremia and fecal virus shedding. There is no detectable seroconversion in the pigs inoculated with the rabbit HEV, even though seroconversion was evident in pigs infected with swine HEV. The lack of seroconversion suggested that infection of pigs by rabbit HEV is not robust, and the

replication level of the rabbit HEV in pigs is low since the estimated GE titer of rabbit HEV in the positive pig fecal samples ranged from 10^3 to 10^5 GE per gram of feces for the US rabbit HEV group, and 10^2 to 10^6 GE per gram of feces for the Chinese rabbit HEV group. The transient and spotty nature of viremia and fecal virus shedding detected in only half of the rabbit HEV-inoculated pigs also indicated an inefficient and low level of rabbit HEV replication in pigs. This is not surprising, considering that this is a cross-species infection and the rabbit HEV will need to adapt to the new host before it can replicate more efficiently. In addition, the relatively low titers of the rabbit HEV virus stocks used for the pig inoculation (2×10^5 - 10^6 GE per ml) may also play a role in the observed low level of rabbit HEV replication in pigs since it is well documented that hepatitis E is a dose-dependent disease (46, 47). It is possible that the rabbit HEV replication level in pigs is not robust and therefore is insufficient to elicit a detectable level of humoral immune response.

To further verify that the pigs are indeed infected by rabbit HEV and excrete infectious virus in the feces, we subsequently inoculated two naïve rabbits with a 10% suspension of PCR-positive pig feces collected from the rabbit HEV-infected pigs. Viremia and fecal virus shedding were consistently detected in the two inoculated rabbits, indicating that the feces from the rabbit HEV-infected pig contain infectious virus. Again, seroconversion to rabbit HEV antibody was not detected in the pig feces-infected rabbits, even though seroconversion was evident in the rabbits experimentally infected with the rabbit HEV USRab-14. Nonetheless the successful transmission of rabbit HEV recovered from feces of inoculated pigs to naïve rabbits further confirmed that the rabbit HEV has the ability to infect pigs.

None of the pigs inoculated with a U.S. rat HEV showed any evidence of infection, as there was no viremia, fecal virus shedding or seroconversion. This is not unexpected as the rat

HEV appears to belong to a new genotype (20, 36). In a recent study, an attempt to infect rhesus monkeys with the rat HEV was also unsuccessful (36). Therefore, the preliminary data from this study suggests that the rat HEV identified from the United States and Germany has a limited host range and is likely not zoonotic, however future studies are warranted to confirm this.

In conclusion, the results from this study demonstrated that the newly-identified rabbit HEV is antigenically related to the other known animal strains of HEV, and that, like other genotype 3 HEV strains, the rabbit HEV also has the ability to infect across species barrier. This raises potential concern of zoonotic transmission of the rabbit strain of HEV to humans through direct contact with infected rabbits or through the consumption of undercooked rabbit meat. Future studies are warranted to determine the zoonotic potential of the rabbit HEV.

FIGURES

Figure 1. Seroconversion to IgG anti-HEV in rabbits experimentally inoculated with the rabbit HEV (USRab-14 strain) recovered from farmed rabbits in Virginia (ID#7 and #8) and with a 10% suspension of RT-PCR-positive feces from a pig experimentally infected with the same strain. The ELISA optical density (OD) values are plotted over weeks post-infection. The rabbits were necropsied at 10 weeks post-infection.

Figure 1.

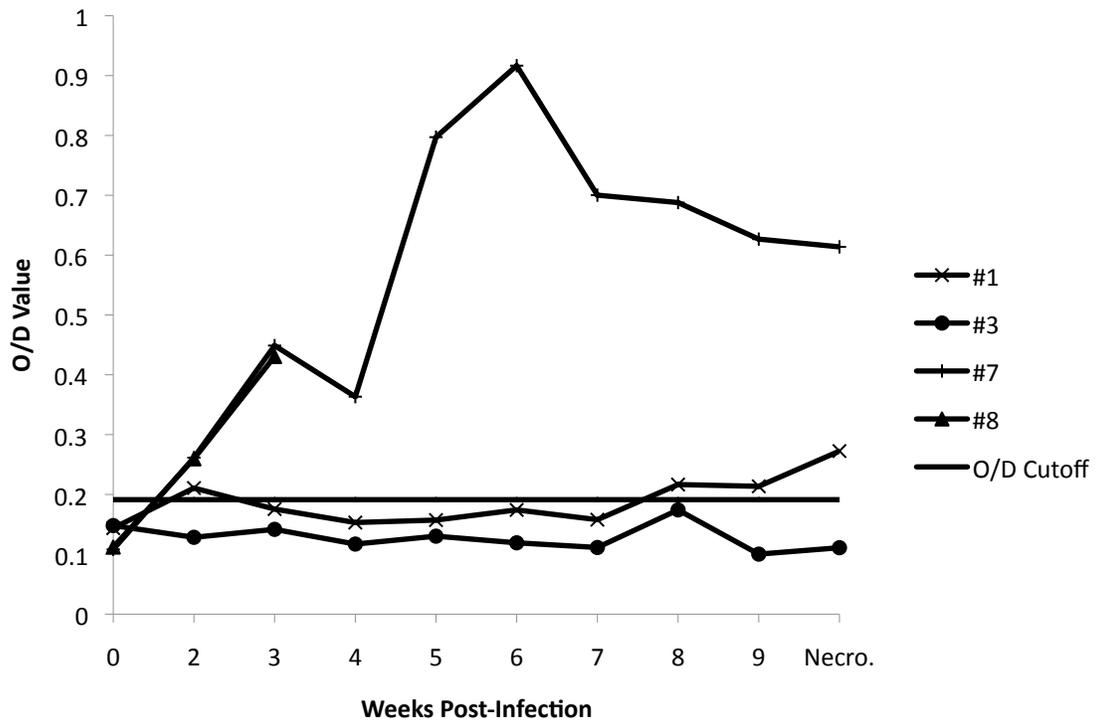
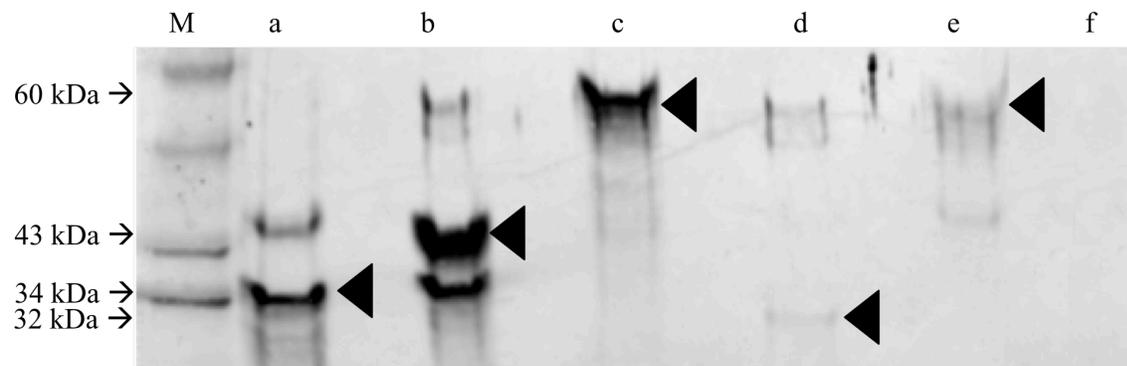


Figure 2. Antigenic cross-reactivity between rabbit HEV antiserum and recombinant capsid antigens from other animal HEV strains by Western blot analysis. **Panel A:** Each lane was loaded with an equal amount (1 μ g) of truncated recombinant capsid proteins derived from different strains of HEV: (a) U.S. rabbit HEV (34kDa); (b) genotype 1 human HEV (43 kDa); (c) genotype 3 swine HEV (60kDa); (d) avian HEV (32kDa); (e) rat HEV (60kDa). The membrane containing each of the recombinant antigens was incubated with a rabbit HEV antiserum. Lane f and M, negative control and molecular marker, respectively. Arrow heads indicate the expected bands of antigen-antibody reaction in the western blot analysis. **Panel B:** Coomassie-staining gel of truncated recombinant capsid proteins derived from different strains of HEV. Each lane was loaded with an equal amount (1 μ g) of either (a) U.S. rabbit HEV (34kDa); (b) genotype 1 human HEV (43 kDa); (c) genotype 3 swine HEV (60kDa); (d) avian HEV (32kDa); or (e) rat HEV (60kDa). Arrow heads indicate the expected sizes of each truncated HEV capsid protein.

Figure 2.

A



B

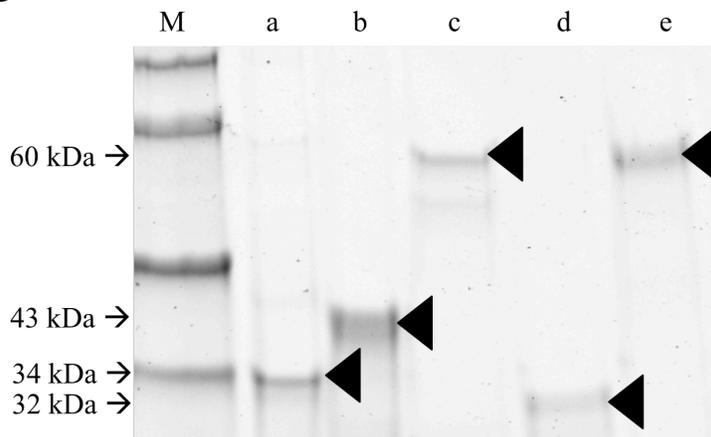
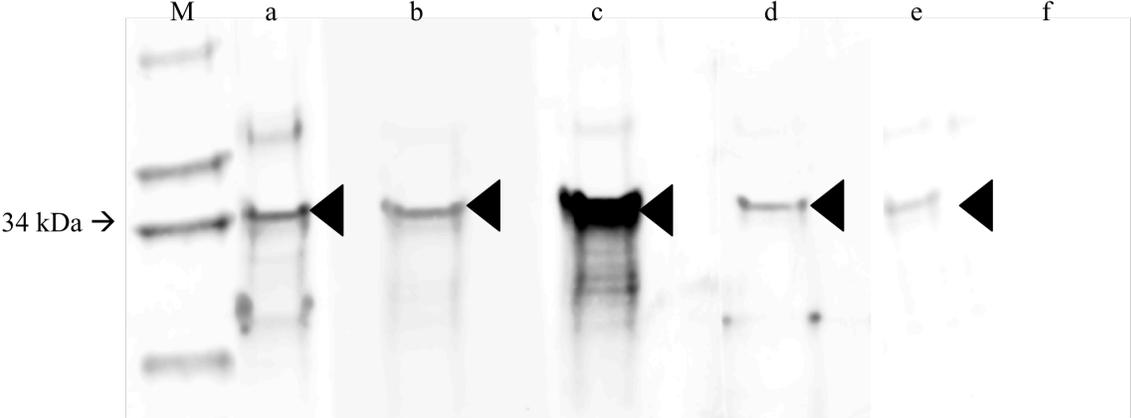


Figure 3. Antigenic cross-reactivity between recombinant rabbit HEV capsid antigen and anti-HEV antisera raised against different animal strains of HEV in a western blot analysis. Each lane was loaded with an equal amount (1 μ g) of the truncated recombinant capsid protein of rabbit HEV (34kDa). The membrane containing the rabbit HEV capsid antigen was incubated with antisera raised against different strains of HEV: (a) rabbit antiserum against U.S. strain of rabbit HEV, (b) swine hyperimmune antiserum from a pig immunized with the capsid antigen of the genotype 1 human HEV, (c) swine antiserum from a pig experimentally infected with genotype 3 swine HEV, (d) chicken antiserum from a chicken experimentally infected with avian HEV, (e) swine antiserum from a pig immunized with the recombinant rat HEV capsid protein. Lane f and M, negative control and molecular marker, respectively. Arrow heads indicate the expected bands of antigen-antibody reaction in the western blot analysis.

Figure 3.



TABLES

Table 1: Oligonucleotides used in RT-PCR detection of respective HEV RNA in this study

Group	Inocula	Primers Used ^a	
1	PBS	USRabdegF1	5'-GCMACACGKTTYATGAARGA-3'
		USRabdegR1	5'-ACYTTRGACCATCVAGRGARC-3'
		USRabdegF2	5'-GCTGAYACRCTTCTYGGYG-3'
		USRabdegR2	5'-TGAMGGRGTRGGYGRTCYTG-3'
2	U.S.Rabbit HEV	USRabF1	5'-GCTGATACGCTTCTTGGTG-3'
		USRabR1	5'-GTGGGCCGATCTTGCTCAT-3'
		USRabF2	5'-ATGGCGAGCCGACTGTCAA-3'
		USRabR2	5'-TCCTGGATAACAACACGGGAAT-3'
3	Chinese rabbit HEV	CHRabF1	5'-TAGCTGATACACTTCTTGGTG-3'
		CHRabR1	5'-CTTAGAGTGCTGCTGGATGGT-3'
		CHRabF2	5'-TCCAGGACTATGATAATCAAC-3'
		CHRabR2	5'-ACTTTAGACCAATCAAGAGAGC-3'
4	Rat HEV	RatHEVF1	5'-GACACACTGCCTGAATGGTG-3'
		RatHEVR1	5'-CGGAGTGCTTCTTCCATCG-3'
		RatHEVF2	5'-ATGGCGATTGTTATGTTTCAGGA-3'
		RatHEVR2	5'-TGGTACAACCTCCACAACA-3'
5	Genotype 3 swine HEV	SwHEVF1	5'-AGCTCCTGTACCTGATGTTGACTC-3'
		SwHEVR1	5'-CTACAGAGCGCCAGCCTTGATTGC-3'
		SwHEVF2	5'-GCTCACGTCATCTGTCGCTGCTGG-3'
		SwHEVR2	5'-GGGCTGAACCAAAATCCTGACATC-3'
-	U.S.Rabbit HEV	RabORF1F1	5'-GAGATGTGTCGCGAATAACG-3'
		RabORF1F2	5'-CCAGGGAATATCAGCTTGGA-3'
		RabORF1R	5'-CCACCGAGTCATCCCCTTTA-3'

^aF1, R1, F2, R2 indicated first round forward, first round reverse, second round forward, second round reverse primers, respectively

Table 2: Fecal virus shedding/viremia in rabbits experimentally inoculated with Chinese rabbit HEV (RC-39) and U.S. rabbit HEV (USRab-14) collected from rabbit and pig feces.

Inocula	Rabbit ID	Positive (+) or negative (-) of HEV RNA detected in fecal samples at indicated week post-inoculation ^a									
		0	1	2	3	4	5	6	7	8	9
RC-39 from rabbit serum	560	-	-	+	+	X ^b					
	562	-	-	+	+	+	+	+	+	+	X ^b
USRab-14 from pig feces	1	-	-	+	-	-	+	+	-	-	-
	3	-	-	-	+	+	+	-	-	-	-
USRab-14 from rabbit feces	7	-	+	+	+	+	+	+	+	+	+
	8	-	+	+	+	+	X ^b				

^aThe fecal samples were collected every other day, and the results are considered positive for that week if at least one of the samples collected during the week tested positive.

^bX: indicates that animal was euthanized prior to test date

Table 3. Fecal virus shedding and viremia in pigs experimentally inoculated with a U.S. strain of rabbit HEV, a Chinese strain of rabbit HEV, a U.S. strain of rat HEV, and a genotype 3 swine HEV

Inocula	Animal ID	Positive (+) or negative (-) of HEV RNA detected in fecal/serum samples at indicated week post-inoculation ^a													
		0	1	2	3	4	5	6	7	8	9	10	L	B	
PBS buffer	1	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	X ^b			
	2	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	-	
	3	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	-	
	4	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	-	
U.S. Rabbit HEV	15	-/-	-/-	-/+	-/-	-/-	-/+	-/-	-/+	-/-	-/-	-/-	-	-	
	16	-/-	-/-	-/-	-/-	-/-	+/-	-/-	-/-	-/-	+/+	-/-	-	-	
	17	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	-	
	18	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	-	
Chinese Rabbit HEV	19	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+	+	
	20	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-	-/-	+/-	+/-	-	+	
	21	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	-	
	22	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	-	
U.S. rat HEV	23	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	-	
	24	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	-	
	25	-/-	-/-	-/-	-/-	-/-	X ^b								
	26	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	-	
Genotype 3 swine HEV	27	-/-	-/-	-/-	+/-	+/-	+/-	+/+	-/-	-/-	-/-	-/-	+	-	
	28	-/-	-/-	-/+	-/-	-/-	-/-	-/-	+/-	+/+	+/-	+/-	+	-	
	29	-/-	+/-	+/-	+/+	+/+	+/-	+/-	-/-	-/-	+/-	+/-	+	-	
	30	-/-	+/-	-/-	+/-	+/-	+/-	+/-	-/-	-/-	+/-	+/-	+	-	

^aL: Liver; B, bile collected during necropsies.

^bX: pigs 1 and 25 died prior to the termination of the project due to circumstances unrelated to the study.

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Chapter 4: Risk Factors and Sources of Foodborne Hepatitis E Virus Infection, United States

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Under Preparation

ABSTRACT

Hepatitis E virus (HEV) is an important human pathogen with pigs and other species serving as animal reservoirs. Swine veterinarians and other pig handlers are at an increased risk of HEV infection. Ample evidence documents sporadic cases of hepatitis E acquired via consumption of undercooked meat. Chronic hepatitis E cases in immunosuppressed individuals are exclusively caused by zoonotic genotype 3 HEV of swine origin. Here we report the identification of HEV from non-liver commercial pork products and association of HEV seropositivity to consumption of undercooked animal meats in the United States. HEV sequences of genotype 3 origin were identified from 2.3% (3/128) of non-liver commercial pork products. Approximately 6.3% (21/335) of college students are seropositive for HEV antibodies with those who had a history of consuming undercooked meats 13 times more likely to be seropositive. The results raise concerns about foodborne HEV transmission in the United States.

Keywords: Hepatitis E Virus (HEV); hepatitis E; foodborne; transmission; zoonosis; risk factors

INTRODUCTION

Hepatitis E virus (HEV), the causative agent of hepatitis E, is an important human pathogen that is endemic in both industrialized and developing countries worldwide (23). Epidemics occur typically in Mexico and in the developing countries of Asia and Africa (21, 22). The epidemics in developing countries are usually associated with HEV-contaminated drinking water due to poor sanitation practices, whereas the sporadic and endemic cases in industrialized countries are typically associated with contaminated food products, and direct contact with infected animals (8, 12, 20, 21, 33). Humans are infected by HEV genotypes 1-4: genotypes 1 and 2 are restricted to human hosts only, while genotypes 3 and 4 have an expanded host range and infect humans and several other animal species (21, 22).

Hepatitis E virus infection in humans typically causes a self-limiting acute hepatitis ranging from subclinical to fulminant hepatic failure (13). Immunocompetent individuals usually clear the virus asymptotically with no complication, while some patients can experience more complicated infections with clinical signs typical of acute viral hepatitis that can include anorexia, jaundice, enlarged liver, abdominal pain, nausea, vomiting, and fever (13, 34). In immunocompromised individuals such as solid organ transplant recipients, patients with concurrent HIV, or other viral hepatitis infections, the disease can progress into chronicity and result in liver cirrhosis and failure to clear the infection (9, 19, 34). Interestingly, these chronic hepatitis E cases are almost exclusively found in industrialized countries and are caused by the zoonotic genotype 3 HEV (34). A hallmark feature of hepatitis E is its relationship with pregnancy and increased maternal and child mortality rates. Reports estimate that the case-fatality risk of HEV-infected pregnant women is at least 20% (30).

Hepatitis E virus infection of humans from contaminated animal meat and direct contact with infected animals has been increasingly documented (21, 33, 35, 38). A 2007 study sampled 125 packages of pork liver collected from local grocery stores in Virginia, and found that 14 (11%) were positive for infectious HEV (14). People with occupational contact with animals are at a higher risk of being seropositive for HEV antibodies than members of the general population (4). Among the 295 swine veterinarians, from eight U.S. states from which age- and geography-matched normal blood donor data was available, 78 (26%) were positive for anti-HEV antibodies, while 73 of 400 (18%) blood donors, without documented history of exposure to swine, from the same eight U.S. states were positive. Swine veterinarians in those eight states were 1.51 times more likely when tested with genotype 3 swine HEV antigen (95% confidence interval, 1.03 to 2.20) and 1.46 times more likely when tested with genotype 1 human HEV antigen (95% confidence interval, 0.99 to 2.17) to be anti-HEV positive than normal blood donors (25). According to the National Health and Nutrition Evaluation Survey (NHANES), the seroprevalence of HEV in the general population in the United States is approximately 6%, only about one-third as high as previously reported (11).

With the increasing evidence of HEV-contaminated commercially-available pork products and cases of zoonotic transmission of humans with genotype 3 or 4 HEV in industrialized countries (2, 10, 27, 31, 33, 37, 39), we were interested in investigating the risk factors associated with HEV seropositivity among an understudied group: otherwise healthy young adults in the United States. The objectives of this study were 1) to compare the anti-HEV seroprevalence among veterinary and university students to the general population in the United States; 2) to identify the potential risk factors associated with HEV seropositivity including age, sex, vegetarian/non-vegetarian lifestyle, and meat temperature preference; 3) to identify the

potential sources of HEV infection from non-liver commercial animal meats products from local grocery stores.

MATERIALS AND METHODS

Serum Sample Collection

Serum samples were collected from 935 from undergraduate and veterinary students at a university in the United States from 2002 through 2009. A survey was distributed to participants asking for age, whether or not they consumed meat, whether or not they ever consumed undercooked meat, and if so how often. Correctly and fully completed surveys could be matched with 335 serum samples. Of these 335 samples, there were samples from 267 female students and 68 male students, 251 veterinary students and 84 undergraduate students, 329 meat consumers and 6 vegetarians, and 224 students who at least occasionally consumed undercooked meat and 124 students who did not (**Table 1**). The mean age of the these 335 participants was 26 years, ranging from 20 years to 45 years of age; 301 students were under 30 years of age and 34 students were older than 30 years of age (**Table 1**). No further personally identifying information was collected.

Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of HEV Antibodies

The Wantai HEV-Ab ELISA kit for *in vitro* qualitative detection of total antibodies to HEV was used in this study (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd., Beijing, China), according to manufacturer's instructions. The HEV research community has generally accepted the Wantai assay as an appropriate commercial assay for HEV serological studies (1).

Quality Control and Interpretation of Serology Results

Each microplate was considered separately, according to manufacturer's instructions, when calculating and interpreting the results of the assay. The results were calculated by relating each sample's optical density (OD) value to the cut-off value (CO) of the plate. The results were calculated, following the Wantai kit instructions, by subtracting the blank well OD value from the print report values of samples and controls. Samples giving absorbance less than the cut-off value ($OD/CO < 1$) were considered negative, indicating that no antibodies to HEV were detected with the kit. Samples giving an absorbance greater than or equal to the cut-off value ($OD/CO \geq 1$) were considered initially reactive, indicating that antibodies to HEV were detected using this kit. Any positive samples were retested in duplicate for confirmation, and repeatedly reactive samples were considered positive for antibodies to HEV.

Statistical analyses

Results were analyzed for a total of 335 serum samples with fully completed survey information available. All variables were first evaluated by a chi-squared test using PROC FREQ of SAS (release 9.3; SAS Institute, Cary, N.C.) to assess univariate associations between risk factors and HEV seropositivity. Variables with model *P*-values of < 0.05 were selected for further introduction into multivariable logistic regression using PROC LOGISTIC of SAS.

Collection and Processing of Commercial Animal Meat Products from Local Grocery Stores

A total of 128 non-liver commercial pork products and 28 packs of commercial rabbit meat products were purchased from six local grocery stores in Southwest Virginia in July and August, 2014. The rabbit meat purchased were individual packs of whole-rabbits with giblets. The commercial pork products purchased from local grocery stores were 12 containers of chitterlings, 15 containers of nervous tissue (including brains and spinal tissue from neck bones),

68 packages of sausage (including ground pork, sausage links, chorizo, bratwurst, longaniza, and sausage patties), and 33 packages of skeletal muscle (including spare ribs, dim sum, pork tenderloin, pork chops, cube steak, chops, and carne enchilada). For each of the separate containers of commercial animal meat products, several 1-gram samples were taken from multiple locations of each meat product in order to get a representation from the entire contents of the package. Each 1-gram sample was then added to 9 ml of Hyclone Phosphate Buffered Saline (Thermo) and homogenized into suspension. All homogenized samples were then stored at -80°C until the RNA extraction procedure was performed.

Nested RT-PCR to Detect HEV RNA from Meat Products

RNA extraction and RT-PCR assembly were performed in a designated, physically separate, Clean Room to avoid potential cross-contamination. RNA extraction was performed on 200 µl of each homogenized pork and rabbit meat samples using a standard Trizol Reagent extraction protocol. Reverse transcription was performed on 12.5 µl of each RNA sample for 60 min at 42°C using 1 µl of the swine or rabbit HEV-specific reverse primer SwDegR1 (5'-CCCTTRTCYTGCTGMGCATTCTC -3') or RabDegR1 (5'-ACYTTRGACCARTCVAGRGARC-3'), respectively, 0.25 µl (20u/µl) Superscript II reverse transcriptase (Invitrogen), 4 µl of 5 x RT Buffer, 1 µl of 0.1 M dithiothreitol, 0.50 µl (40u/µl) RNase inhibitor (Promega), and 1 µl of 10 mM deoxynucleoside triphosphates. Five microliters of the resulting cDNA was then amplified in a 50µl PCR reaction with Platinum PCR SuperMix High Fidelity (Life Technologies) using the swine or rabbit HEV-specific primers. For the first round of nested PCR, external primers SwDegF1 (5'- AATTATGCYCAGTAYCGRGTTG-3') and SwDegR1 (5'- CCCTTRTCYTGCTGMGCATTCTC -3') were used for all pork samples, while primers RabDegF1 (5'-GCMACACGKTTYATGAARGA-3') and RabDegR1 (5'-

ACYTTRGACCARTCVAGRGARC-3') were used for all rabbit meat samples. For the second round of PCR internal primers, primers SwDegF2 (5'-GTWATGCTYTGCAWCAWGGCT-3') and SwDegR2 (5'-AGCCGACGAAATCAATTCTGTC-3') were used for pork meat samples, and RabDegF2 (5'-GCTGAYACRCTTCTYGGY-3') and RabDegR2 (5'-TGAMGGRGTRGGMYGRTCYTG-3') were used for all rabbit meat samples. The PCR parameters used in this study were essentially the same as described previously (17). Following nested RT-PCR amplification, samples were run on a 1.5% agarose gel. If positive PCR bands were apparent, the PCR products were then purified following standard gel extraction protocol (Qiagen), and subsequently sequenced to verify the authenticity of the amplified products.

Positive and negative controls were included in all rounds of RNA extraction, reverse transcription, nested PCR, and gel extraction to ensure the integrity of any positive RT-PCR results. The positive controls were samples previously collected from pigs experimentally infected with genotype 3 swine HEV, and rabbits experimentally infected with the U.S. rabbit HEV (7). Commercial meat product samples were considered positive for HEV RNA if the amplified PCR products represented unique HEV sequences.

Phylogenetic Analyses of HEV Sequences Amplified from Commercial Pork Products

Hepatitis E virus sequences spanning a 245-nt region in the ORF2 from all amplified PCR products were analyzed. Sequence alignment was completed using ClustalW, MEGA version 6.0 was used (www.megasoftware.net) for the phylogenetic analysis. The tree was constructed using the neighbor-joining method with the 1000 bootstrap analyses (**Figure 1**). In total, three HEV positive samples were sequenced, subsequently analyzed, and their sequences deposited in GenBank (GenBank accession numbers: KR108015 to KR108017).

RESULTS

Seroprevalence of HEV Antibodies is Dependent on Age and Consumption of Undercooked Meats in Healthy College Students

The overall prevalence of anti-HEV antibodies was 6.27% (21/335). Seropositivity was associated with age ($P=0.0407$) and consumption of undercooked meat ($P=0.0157$) in univariate analysis, while there was no association ($P>0.1$) of seropositivity and gender, student status (veterinary or undergraduate) or lifestyle (meat consumer or vegetarian) (**Table 1**). Effects of age and of consumption of undercooked animal meats remained significant when both variables were introduced into a multivariable logistic regression model. The odds of subjects older than 30 years being positive for anti-HEV antibodies was 3.14 times the odds of those younger than 30 years of age (95% confidence interval, 1.036 to 9.508), and the odds of subjects who, when surveyed, answered that they “always” or “occasionally” consumed undercooked meat to be positive for anti-HEV antibodies being 13.0 times (95% confidence interval, 1.717 to 98.450) the odds of those who answered that they “never” consumed undercooked meat (**Table 2**).

Detection of HEV RNA in Non-liver Commercial Pork Products from Grocery Stores

Hepatitis E virus RNA was detected in 3 out of 12 packages (25%) of pork chitterlings purchased from grocery stores in Southwest Virginia, giving a total prevalence of HEV RNA of 3 out of 128 (2.3%). HEV RNA was not detected in any of the packaged rabbit meat products or non-chitterling cuts of pork tested in this study.

HEV Sequences Detected in Commercial Pork Products Belong to the Zoonotic Genotype 3

All HEV RNA-positive samples were sequenced to confirm the authenticity. Among the positive pig meat samples, there were two unique HEV sequences. The other positive PCR

product was isolated from separate packages of meat, but represents HEV sequences identical to one unique sequences. This is likely because the meat from these samples was purchased at the same store on the same day, so may have contained tissues from the same herd of pigs. Sequence analyses revealed that both of the HEV sequences amplified from the pork products in this study belong to the zoonotic genotype 3. The two HEV sequences amplified from non-liver commercial pork products share approximately 90% nucleotide sequence identity to each other, and approximately 88-97% sequence identity to existing strains of genotype 3 HEV from both humans and pigs. A phylogenetic tree was also constructed to depict the relationship of the HEV isolates from this study to the existing strains of HEV, and both HEV isolates cluster within the genotype 3 (**Figure 1**).

DISCUSSION

Hepatitis E virus differs from other human hepatitis viruses in that it has animal reservoirs, as initially documented with the discovery of swine HEV in pigs in 1997 (24). Genetic identification of HEV strains from various other animal hosts and the demonstration of cross-species transmission of some HEV strains have broadened the host range and genetic diversity of the virus. Thus far, unique strains of HEV have been identified from humans, pigs, wild boars, chickens, rabbits, deer, rats, ferrets, mongoose, bats, and fish (16, 18, 21, 23, 24, 28, 32, 39, 40). Mammalian HEV has been documented to cause acute hepatitis in humans and has known animal reservoirs (21). There are currently four recognized HEV genotypes that infect humans within the genus *Hepevirus* of the family *Hepeviridae*. Genotypes 1 and 2 are human viruses that have been identified as causing epidemic acute hepatitis E and are associated with waterborne and fecal-oral transmission in developing countries, while genotypes 3 and 4 have

been identified in humans and other animal species, are zoonotic, and are endemic in both industrialized and developing countries (20, 21, 33).

Pigs are now recognized animal reservoirs of HEV and as sources of transmission to humans. Consumption of undercooked pig meat has been increasingly linked to cases of hepatitis E throughout the world (2, 5, 26, 27, 29, 33, 35, 37). Detectable levels of HEV RNA were found in 11%, 4%, and 6.5% of tested commercial pork liver samples in the United States, Germany, and the Netherlands, respectively (3, 14). Pig liver consumption, in the United States and other industrialized countries, however, is not common, and thus the relatively high seroprevalence of anti-HEV antibodies in these countries suggests other potential sources of infection. Unfortunately, up until this point, there have been no reports on the presence of HEV in non-liver commercial pork products in the United States.

It has been demonstrated that individuals with occupational contact with animals are at a higher risk of being seropositive for HEV antibodies than the general population (4). For example, swine veterinarians in the United States are at a higher risk of HEV infection compared to age- and geography-matched blood donor controls (25). We therefore were interested in determining the anti-HEV seroprevalence rates in veterinary and undergraduate university students (**Table 1**).

We found that the overall anti-HEV prevalence of 6.27% in veterinary and undergraduate students from this study was approximately that of the estimated nationwide anti-HEV prevalence of 6% as determined by a 2014 NHANES study (11). We also demonstrated an age-linked risk of HEV infection with participants older than 30 years of age being about three times

more likely to be positive for anti-HEV antibodies than those younger than 30 years of age (**Table 2**).

Importantly, we found that consumption of undercooked meat is a risk factor for HEV seropositivity among young adults in the United States, with subjects who consumed undercooked or raw animal meats 13 times more likely to be seropositive than those who did not. The results suggested that eating under-cooked animal meats represent a source of foodborne zoonotic HEV infection in the United States.

Some study limitations must be considered in the interpretation of the study results: only undergraduate college and veterinary students were included in the sample, and so it is not a general population study. Additionally, as it was a retrospective study, the original survey that was designed for a different study did not include questions on risk factors that have been associated with HEV infection such as consumption of contaminated shellfish or produce and travel outside of the United States (12, 35). Despite these limitations, however, this study identified that consumption of undercooked meat is a risk factor that increases with age in young adults.

Our finding in this study of an association between undercooked meat consumption and HEV seropositivity prompted us to investigate other possible non-liver sources of HEV infection in the United States. Since the zoonotic genotype 3 HEV has been identified from pigs and rabbits in the United States, we tested for the presence of HEV RNA in pork and rabbit meat products from local grocery stores (6, 24). We report here, for the first time, the identification of HEV RNA from non-liver commercial pork products in the United States. Hepatitis E virus sequences were identified from 3 of 12 (25%) packages of pork chitterlings, which raised a

concern over potential foodborne HEV transmission via consumption of undercooked pork products, or contaminated surfaces during the preparation of pork dishes. Extrahepatic sites of HEV replication have been reported in gastrointestinal tissues, mesenteric lymph nodes, and spleen (36), and thus it is not surprising to detect HEV RNA in non-liver pork products such as chitterlings. It has been reported that, in order to sufficiently inactivate HEV, meat should be cooked to a minimum internal temperature of 71°C (15). Therefore, under-cooked pork dishes served from medium-to-rare cooking conditions in restaurants could represent a potential source of foodborne HEV infection in humans.

Although HEV RNA was not detected from commercial rabbit meat from grocery stores in this study, our previous research has identified that commercially farmed rabbits in the United States are infected with the zoonotic genotype 3 HEV (6). Additional studies with a large number of commercial rabbit products from different sources are warranted to further evaluate commercial rabbit meat as a potential source of foodborne HEV infection in the United States. HEV RNA was also not detected from other non-chitterling cuts of pork in this study, however, due to previous reports of HEV RNA from commercial pork livers, as well as our identification of HEV RNA in chitterlings, more studies are essential to investigate the presence of HEV in commercial pork, such as sausages, that are likely to contain HEV-infected tissues.

The results from this study underscore the importance of thoroughly cooking meat products, particularly pork. The fact that the increasing numbers of cases of chronic hepatitis E in immunocompromised individuals are almost exclusively caused by the zoonotic genotype 3 HEV of swine origin has raised serious pork safety concerns over HEV transmission (34). Healthy individuals such as those surveyed in this present study, when exposed to HEV via consumption of under-cooked meats, may only develop subclinical HEV infection with

seroconversion to anti-HEV antibodies and no overt clinical disease. Immunocompromised individuals such as solid organ transplant recipients, however, when exposed to HEV via consumption of under-cooked meats may be unable to effectively clear the infection and thus might develop more serious chronic hepatitis E. More studies are warranted to raise the awareness on the risks associated with consuming undercooked meats.

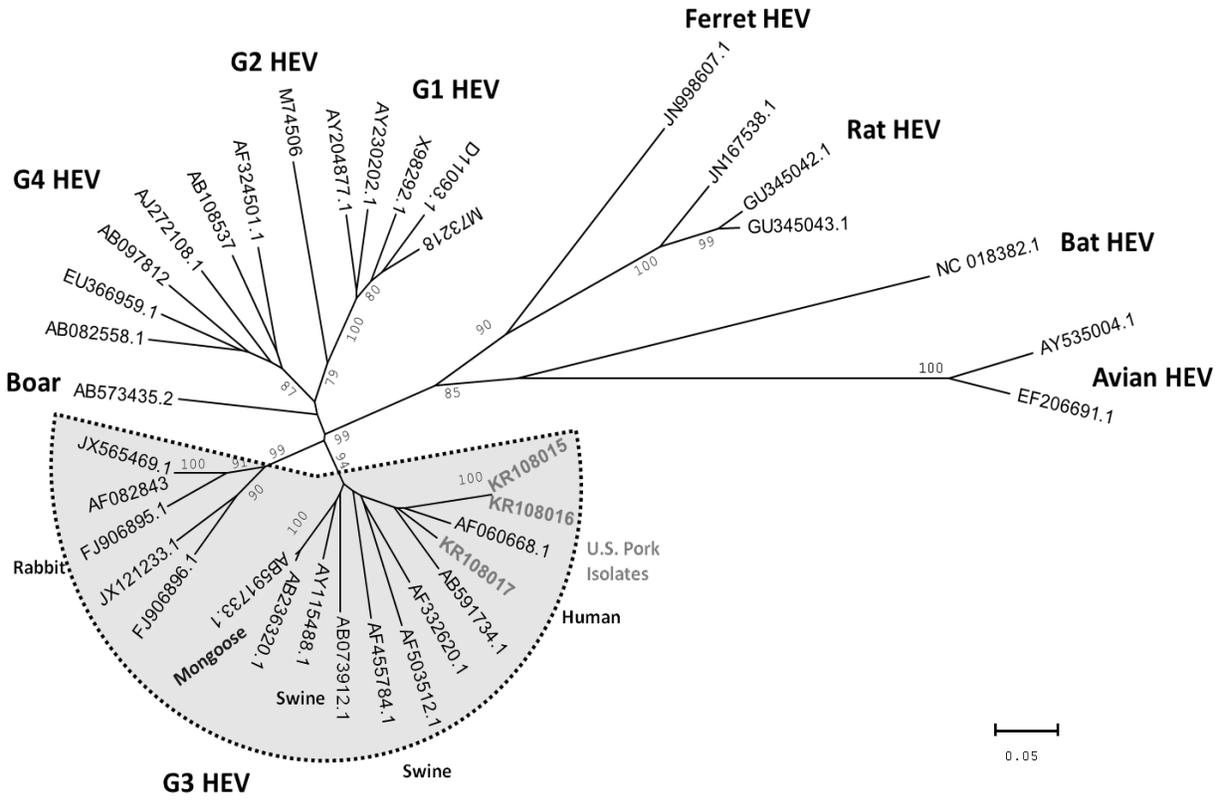
ACKNOWLEDGEMENT

This study was supported in part by grants (R01AI050611 and R01AI074667) from the US National Institutes of Health.

FIGURES

Figure 1. A phylogenetic tree was constructed based on the 245-nt region of the HEV ORF2 capsid gene amplified from commercial pork meat products in Southwest Virginia grocery stores and other representative strains from various HEV genotypes. Existing HEV strains and novel HEV isolates from commercial pork are identified by their accession numbers. Each of the four known genotypes (G1-G4) of HEV is labeled, with representative strains included for each species, and novel strains from wild boar, ferret, rat, avian identified individually as separate putative genotypes. The scale bar represents 0.05 nucleotide substitutions per position, and the bootstrap values greater than 80% are labeled at the major nodes.

Figure 1.



TABLES

Table 1. Risk factors associated with HEV seropositivity in undergraduate and veterinary students; univariate analysis.

Risk Factor	No. tested	No. positive (%)	OR	95% confidence interval	P value
(Overall prevalence)	335	21 (6.27)	-	-	-
Age					
≥ 30 years	34	5	3.071	1.049 – 8.993	*0.0407
< 30 years	301	16			
Gender					
Male	68	4	0.919	-	0.8830
Female	267	17			
Student status					
Veterinary	251	15	0.826	-	0.7029
Undergraduate	84	6			
Lifestyle					
Meat consumer	329	21	0.906	-	0.9470
Vegetarian	6	0			
Undercooked meat consumed					
Yes	224	20	12.059	1.598 – 90.979	*0.0157
No	124	1			

** $P < 0.05$ indicates significant association

Table 2. Risk factors associated with HEV seropositivity; multivariate stepwise logistic regression analysis of variables with $P < 0.05$ when analyzed by univariate analysis.

Risk Factor	OR	95% confidence interval	<i>P</i>-value
Age	3.138	1.036 – 9.508	*0.0431
Undercooked meat consumed	13.000	1.717 – 98.450	*0.0130

* P -value < 0.05 indicates significant association

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Chapter 5: General Conclusions

In this dissertation research, a novel strain of hepatitis E virus was discovered for the first time from rabbits in the United States. Determination of its complete genomic sequence allowed for in-depth sequence and phylogenetic analyses. The novel U.S. rabbit HEV was found to be genetically related to existing strains of zoonotic genotype 3 HEVs, and therefore posed a potential risk to food safety and public health. This concern was further verified when we subsequently demonstrated that the U.S. rabbit HEV cross reacted antigenically with other existing strains of HEV and could also cross species barriers and infect pigs, the known animal reservoir of zoonotic genotype 3 HEV.

Domestic pigs have been recognized as natural animal reservoirs of zoonotic HEV, and there are increasing reports of human infection of HEV through the consumption of undercooked animal meats. In the United States, there have been no reports on the other commercial animal meat sources other than pork liver as a risk of HEV infection. Because significant proportions of the United States populations do not regularly consume dishes containing pork meat, we investigated other possible sources of HEV infection in the U.S. food supply and also attempted to identify potential risk factors for infection.

We reported for the first time the identification of HEV from non-liver commercial pork products and also an association of HEV seropositivity to consumption of undercooked animal meats in otherwise healthy young adults in the United States. The results from this study further emphasize the importance of cooking meat, particularly pork products, thoroughly and using proper hygiene when preparing meals. Taken together, the research in my PhD dissertation has further demonstrated hepatitis E virus as an important foodborne disease in the United States.

We have illuminated the need for more research to further investigate the molecular determinants of cross-species transmission and pathogenicity of the virus, as well as to raise the awareness on the potential zoonotic risk and public health implications of these novel animal strains of HEV in the United States food supply.