Pathogenesis and Cross-species Infection of Hepatitis E Virus

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ABSTRACT

Hepatitis E Virus (HEV), the causative agent of hepatitis E, is a zoonotic pathogen of worldwide significance. The genus Orthohepevirus A of the family Hepeviridae includes all mammalian strains of HEV and consists of 8 recognized genotypes. Genotypes 1 and 2 HEVs only infect humans and genotypes 3 and 4 infect humans and several other animal species including pigs and rabbits. An ever-expanding host range of genetically-diversified strains of HEV now include bat, fish, rat, ferret, moose, wild boar, mongoose, deer, and camel. Additionally, the ruminant species goats, sheep, and cattle have been implicated as potential reservoirs as well.

My dissertation research investigates a novel animal model for HEV, examines the immune dynamics during acute infection, and evaluates the possibility of additional animal reservoirs of HEV. The first project established an immunoglobulin (Ig) heavy chain knock-out JH (−/−) gnotobiotic piglet model that mimics the course of acute HEV infection observed in humans and evaluated the pathogenesis of HEV infection in this novel animal model. The dynamics of acute HEV infection in gnotobiotic pigs were systematically determined with a genotype 3 human strain of HEV. We also investigated the potential role of immunoglobulin heavy-chain JH in HEV pathogenesis and immune dynamics during the acute stage of virus infection. This novel gnotobiotic pig model will aid in future studies into HEV pathogenicity, an aspect which has thus far been difficult to reproduce in the available animal model systems.

The objective of the second project for my PhD dissertation was to determine if cattle in the United States are infected with a bovine strain of HEV. We demonstrated serological evidence of an HEV-related agent in cattle populations with a high level of IgG anti-HEV prevalence. We
demonstrated that calves from a seropositive cattle herd seroconverted to IgG binding HEV during a prospective study. We also showed that the IgG anti-HEV present in cattle has an ability to neutralize genotype 3 human HEV in vitro. However, our exhaustive attempts to detect HEV-related sequence from cattle in the United States failed, suggesting that one should be cautious in interpreting the IgG anti-HEV serological results in bovine and other species. Collectively, the work from my PhD dissertation delineated important mechanisms in HEV pathogenesis and established a novel animal model for future HEV research.


**Pathogenesis and Cross-species Infection of Hepatitis E Virus**

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**GENERAL AUDIENCE ABSTRACT**

Hepatitis E Virus (HEV), the causative agent of hepatitis E, is a zoonotic pathogen of worldwide significance. According to the World Health Organization, there are approximately 20 million HEV infections annually, which result in 3.3 million cases of acute hepatitis E and >44,000 HEV-related deaths. Hepatitis E is a self-limiting acute disease in general, but carries the ability to cause high mortality in pregnant women and chronic hepatitis in immunocompromised individuals. The underlying mechanisms of HEV host tropism and progression of disease to chronicity are unknown.

My dissertation work investigates a novel animal model for HEV, evaluates the possibility of additional animal reservoirs of HEV, and examines the immune dynamics during acute infection. The first project established an immunoglobulin (Ig) heavy chain knock-out J_H (-/-) gnotobiotic piglet model that mimics the course of acute HEV infection observed in humans. The dynamics of acute HEV infection were determined in both the knock-out and wild-type piglets with a genotype 3 strain of human HEV. We also investigated the potential role of immunoglobulin heavy-chain J_H in HEV pathogenesis and virus infection. In the second project, we determined if cattle in the United States are infected with a bovine strain of HEV. We showed serological evidence of an HEV-related agent in cattle as well as calves born in a seropositive herd. Despite the detection of specific antibodies recognizing HEV in cattle, definitive evidence of virus infection could not be demonstrated. Our exhaustive attempts to detect HEV-related sequence from cattle in the United States failed, suggesting that one should be cautious in interpreting the IgG anti-HEV serological results in bovine and other species. Collectively, the work from my PhD
dissertation research delineated important mechanisms in HEV pathogenesis and established a novel animal model for future HEV research.
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DEDICATION

For my daughter, Vienna.
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GENERAL INTRODUCTION

Hepatitis E virus (HEV), the causative agent of hepatitis E, is a fecal- orally transmitted virus (1) and an important disease of public health concern worldwide (2-4). In developing regions of the world, poor sanitary conditions lead to acute outbreaks due to contaminated food or water (5, 6), while here in the United States and other industrialized countries, sporadic and autochthonous cases of hepatitis E prevail (7, 8). Mortality rates for acute infections range from 0.5-4% with the majority of immunocompetent individuals clearing the infection with limited clinical signs (5). However, HEV-infected pregnant women may reach a mortality rate of 28-30% and experience complications including death of the fetus and mother, abortion, and premature birth (9, 10). Likewise, immunocompromised individuals (11) such as solid organ transplant recipients (12-14) or those with concurrent HIV/AIDS (15), other viral hepatitis infections (16), or lymphosarcoma (17, 18) experience progressive disease with the development of chronic HEV infections. Recently, progressive hepatitis E disease has emerged in a new extrahepatic manifestation as a neurological disorder (19-23). The underlying mechanisms leading to increased severity of disease and hepatic damage in these populations are not well understood and necessitate the identification of animal models to replicate all outcomes of HEV infection.

HEV is a single-stranded, positive-sense RNA virus classified as a *Hepevirus* in the family *Hepeviridae* (24), which consists of eight genotypes, including four genotypes affecting the human population. The genus *Orthohepevirus A* includes genotypes 1 and 2 that infecting humans (25), and genotypes 3 and 4 that are zoonotic in nature and infect humans (4, 5) and a wide range of animal species including swine (26-29), and rabbits (30-32). Numerous animal species serve as natural hosts and reservoirs for HEV with genetic identification in rats (33, 34), wild and domestic swine (35, 36), mongoose (37), rabbits (30-32), chickens (38-40), ferrets (41), cutthroat trout (42),
bats (43), deer (35, 44, 45), moose (46), and camel (47), with an ever-expanding host range. However, swine remain the primary reservoir for HEV (48, 49) and zoonotic genotype 3 HEVs are definitely linked to human cases through direct contact with infected animals (50-52) or consumption of contaminated animal products (2, 53-56). Ruminant species including bovine (57-59), sheep (60, 61), and goats (62) have been implicated as reservoirs due to the identification of IgG antibodies binding to HEV in serum; however, the virus has not yet been definitively genetically identified from these species. The ability to control HEV relies on identifying all reservoir species and methods of transmission and addressing the effect on the human food chain. Additionally, the lack of an adequate animal model to assess pathogenesis of disease, the course of infection seen in humans infected with HEV, and immune parameters important during infection led the focus for this dissertation.
REFERENCES


Chapter I: Hepatitis E Virus: Foodborne, Waterborne, and Zoonotic Transmission

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ABSTRACT

Hepatitis E virus (HEV) is responsible for epidemics and endemics of acute hepatitis in humans mainly through waterborne, foodborne, and zoonotic transmission routes. HEV is a single-stranded, positive-sense RNA virus classified in the family Hepeviridae and encompasses four known genotypes (1-4), at least two new putative genotypes of mammalian HEV, and one floating genus of avian HEV. Genotypes 1 and 2 HEVs only affect humans, while genotypes 3 and 4 are zoonotic and responsible for sporadic and autochthonous infections in both humans and several other animal species worldwide. HEV has an ever-expanding host range and has been identified in numerous animal species. Swine serve as a reservoir species for HEV transmission to humans; however, it is likely that other animal species may also act as reservoirs. HEV poses an important public health concern with cases of the disease definitively linked to handling of infected pigs, consumption of raw and undercooked animal meats, and animal manure contamination of drinking or irrigation water. Infectious HEV has been identified in numerous sources of concern including animal feces, sewage water, inadequately-treated water, contaminated shellfish and produce, as well as animal meats. Many aspects of HEV pathogenesis, replication, and immunological responses remain unknown, as HEV is an extremely understudied but important human pathogen. This article reviews the current understanding of HEV transmission routes with emphasis on food and environmental sources and the prevalence of HEV in animal species with zoonotic potential in humans.

Keywords: hepatitis E virus; HEV; zoonosis; animal reservoir; foodborne transmission; zoonotic transmission; waterborne transmission
1. Introduction

Hepatitis E virus (HEV), the causative agent of hepatitis E in humans, is an important public health disease in many parts of the world [1,2,3,4]. Transmission is primarily via fecal-oral route through contaminated food or water [5]. In developing countries in Asia and Africa, poor sanitation conditions lead to outbreaks of acute hepatitis E; however, sporadic and autochthonous cases of hepatitis E also occur throughout many industrialized countries in Europe, Asia, and North America [6,7]. In humans, the mortality rate ranges from 0.5–4% for immunocompetent individuals, however, mortality in HEV-infected pregnant women can reach up to 20% and immunocompromised individuals may develop a chronic HEV infection [8,9]. In addition to humans, HEV has been identified in numerous other animal species including wild and domestic swine, deer, chicken, mongoose, rat, ferret, fish, and rabbits with an ever-expanding host range [1,7,10]. Hepatitis E is now a recognized zoonotic disease with swine and likely other animals serving as the reservoir for human infections [1,8]. Food safety associated with HEV contamination is an important public health concern with the recent identification of infectious HEV in meat and meat products and resultant sporadic cases of foodborne hepatitis E in the human population [3,11,12,13,14]. This review article discusses the public and environmental health concerns and risks associated with HEV infection with an emphasis on foodborne and zoonotic transmissions.

2. HEV Classification and Biology

2.1. Classification

HEV belongs to the genus *Hepevirus* in the family *Hepeviridae* and consists of four recognized genotypes and at least two putative new genotypes [5]. Genotype 1 causes large outbreaks of acute
hepatitis E in humans in Asia. Genotype 2 causes outbreaks in humans and includes one Mexican strain and several African strains. Genotype 3 is associated with sporadic, cluster, and chronic cases of hepatitis E in humans, mostly in industrialized countries. Genotype 3 HEV is known to be zoonotic and has also been isolated from domestic and wild swine, deer, mongoose, rats, and rabbits [12,15,16,17,18,19]. Genotype 4HEV is also zoonotic and is associated with sporadic cases of hepatitis E in humans and infects wild and domestic swine and reportedly cattle and sheep [1,5].

Avian HEV from chickens only shares approximately 50% nucleotide sequence identity with mammalian HEV; therefore, avian HEV likely represents a separate genus [20]. The genus Avihepevirus has recently been proposed to include all three known genotypes of avian HEV in chickens (genotype 1 in Australia and Korea, genotype 2 in the United States, and genotype 3 in Europe and China) [1,21,22]. The recently-identified rat HEV shares approximately 59.9% and 49.9% sequence identities with human and avian HEV, respectively, while the ferret HEV shares the highest sequence identity with rat HEV at 72.3% [18,23]. The genus Orthohepevirus has recently been proposed to encompass both the rat and ferret strains of HEV as well as a novel wild boar HEV strain recovered in Japan that differed from the known genotypes 1-4 HEV isolates by 22.6–27.7% in nucleotide sequence identity [1,24]. A bat HEV was recently identified from African, Central American, and European bats, and due to high sequence diversification from known HEV isolates at 47% amino acid sequence identity, the bat HEV forms a novel phylogenetic clade [25]. The genus Chiropteranhepevirus has been proposed to include all variants of the bat HEV [1]. Finally, a strain of HEV was also identified in cutthroat trout in the United States with only 13–27% sequence homology with mammalian or avian hepeviruses leading to a proposal of another tentative genus, Piscihepevirus, within the Hepeviridae family [1,26]. The nomenclature
of HEV will need to be modified in the near future as more genetically-divergent animal strains of HEV are identified.

2.2. HEV Biology

The genome of HEV is a single-stranded, positive-sense, RNA molecule of approximately 7.2kb in size [3,4,27]. The genome consists of three open reading frames (ORFs), a 5’ non-coding region (NCR), and a 3’ NCR [10]. ORF1 encodes non-structural proteins with conserved domains functioning as a methyltransferase, helicase, RNA-dependent RNA polymerase (RdRp), and a papain-like cysteine protease [20,28]. In addition, a hypervariable region (HVR) within ORF1 may play a role in viral pathogenesis despite being shown to have no influence on viral infectivity [29]. ORF2 encodes the immunogenic capsid protein, which interacts with 3’ viral genomic RNA for encapsidation and contains an endoplasmic reticulum signal peptide and 3 N-glycosylation sites [30,31]. ORF3 encodes a small phosphoprotein with incompletely understood functions; however, the association with cytoskeleton and its necessity for in vivo viral infection in rhesus macaques suggests that ORF3 plays a role in viral replication and assembly [20,32,33].

Avian HEV is genetically related to mammalian HEV with conserved genomic organization and function despite a 600 bp sequence deletion [34,35,36]. The capsid protein of avian HEV contains both unique and conserved antigenic epitopes in comparison to the human and swine HEV capsid proteins [37].

The HEV replication cycle is currently not well understood due to a lack of an efficient cell culture system [38]. Heparin sulfate proteoglycans (HSPGs) likely act as receptors for the attachment of the viral capsid protein, and the heat shock cognate protein 70 may be involved in HEV entry into the cell [38]. Following uncoating in the cell, the HEV genomic RNA is likely utilized to translate the non-structural proteins and the viral RdRp is used to produce progeny
virus [38]. Both ORF2 and ORF3 are translated from a bicistronic subgenomic RNA [32,39]. The negative-sense HEV RNA indicative of virus replication is detectable in hepatic and extrahepatic tissues of experimentally-infected rhesus macaques and swine [38,40]. Post-translational processing of proteins and mechanisms of virus assembly and release have yet to be fully elucidated, and the viral-host interactions leading to a disease state are also poorly understood [5,20,38]. Development of a robust cell culture system to efficiently propagate HEV in the future should be a priority, and will facilitate our understanding the biology of this important virus.

3. HEV Pathogenesis

3.1 HEV infection in humans

In humans, HEV causes an acute icteric disease that varies in symptoms from subclinical to fulminant hepatitis [4]. The asymptomatic patient typically clears the virus rapidly, while the symptomatic patient experiences clinical signs including anorexia, hepatomegaly, myalgia, jaundice and sometimes abdominal discomfort, nausea, vomiting, and fever [5,41]. In immunocompromised patients such as organ transplant recipients, lymphoma and leukemia patients, or patients with HIV infection, the course of disease may progress to a chronic state with cirrhosis of the liver and persistence of viral shedding [42,43,44,45,46]. Of particular concern is the ability for HEV-infected immunocompromised individuals to develop clinical disease well after the initial exposure [44,45,46,47]. Currently, chronic HEV infection in immunocompromised individuals is an emerging and significant clinical problem. Future studies are warranted to identify the immunological correlates and host factors leading to chronicity.
The typical infection begins with an incubation period of 2 weeks to 2 months and a transient viremia followed by viral shedding in the feces, disappearance of viremia with the onset of clinical signs, and regression of viral shedding with potential jaundice setting in around 2-3 weeks into the infection [46]. The severity of HEV infection is considered dose-dependent and host factors such as concurrent hepatic disease or alcohol overuse may also contribute to the disease course [41]. In studies from France, Germany, the United Kingdom, and the United States, middle-aged, elderly men were more likely to experience autochthonous HEV infection; however, the underlying host factors have not been understood [48,49,50,51]. Of major concern is the relationship between pregnancy and increased mortality rates up to 20% in HEV endemic regions; however, this relationship appears to be geographically dependent and may be associated with other underlying factors such as virus genotype or concurrent infections with other pathogens [4,20,52,53,54]. Complications with concurrent HEV infection during pregnancy include death of both the mother and fetus, abortion, premature birth, and death of the baby shortly after birth [55]. Vertical transmission from the mother to fetus was reported in 33% of cases and HEV RNA was reportedly detected in human colostrum as well [56,57]. Unfortunately it is not understood why pregnancy resulted in severe hepatitis E manifestation. Understanding the mechanisms of pregnancy-associated severe hepatitis E, especially fulminant hepatitis E, in the future will help devise effective preventive measures against this disease.

Genotypes 1 and 2 HEV strains are restricted to the human population, while genotypes 3 and 4 HEV strains infect both humans and other animals with zoonotic transmission routes. Human to human transmission of HEV is considered rare; although, blood-borne transmission has been reported via blood transfusion [20,58,59,60]. A comparative study of genotype 3 and 4 HEV-infected individuals in Japan revealed that genotype 4 HEV is associated with a higher level of
alanine aminotransferase (ALT), higher prevalence of clinical infection, higher level of total bilirubin, higher level of viremia, more frequent fulminant hepatitis development, and overall a more aggressive hepatitis [48]. The mechanisms of cross-species HEV infection remain poorly understood. Identification of both the viral genetic elements and host factors that are important for cross-species HEV infection will be the key for devising strategies to prevent and control zoonotic HEV infections.

3.2 HEV infection in animals

Natural and experimental HEV infections in swine (genotypes 3 and 4) result in a subclinical course of infection with only mild microscopic lesions in the liver and associated lymph nodes [61,62]. Viremia lasts 1–2 weeks with fecal virus shedding lasting 3–7 weeks [7,61,62]. HEV infection in swine is age-dependent with up to 86% of the pigs infected by 18 weeks of age [63]. Additional studies from the United Kingdom, Spain, and Japan further demonstrated that the highest fecal virus shedding occurred by 10–12 weeks, 13–16 weeks, and 1–3 months of age, respectively [64,65,66]. Seroconversion to HEV antibodies in swine occurs following the typical waning in maternal antibody levels around 8–10 weeks of age first with IgM anti-HEV antibodies peaking in conjunction with fecal viral shedding followed by IgG anti-HEV antibodies peaking in conjunction with clearance of virus from the feces [20,64,65,66]. Transmission between swine is fecal-oral with large amounts of infectious HEV being shed in the feces, and direct contact between animals, with other animals’ excreta, and with potentially contaminate and water sources in swine facilities contributes to transmission within a herd [7,67,68,69,70]. Although HEV infection in pigs does not pose a major economical concern in swine production, the risk of zoonotic transmission to humans is an important public health concern. Therefore, development of an
effective vaccine to immunize susceptible swine herds in the future will minimize the risk of zoonotic infection and improve pork safety.

Avian HEV genotypes 1-3 carry a slightly different course of infection with a high level of subclinical infection in flocks and mortality rates up to 0.3–1.0% [36,71,72]. Clinical signs may include egg drop in some flocks up to 20%, enlargement of the liver and spleen, and acute death of affected birds [73]. Post-mortem evaluations show enlarged, hemorrhagic, and focally necrotic livers, inflammatory cellular infiltrations in the liver tissue, serosanguinous abdominal fluid, and regressing ovaries in some affected birds [73,74]. It appears that avian HEV does not infect humans, and thus is not a concern for food and environmental safety. Nevertheless, more studies are needed to fully assess the potential of avian HEV cross-species infection.

4. Epidemiology of HEV Infection

HEV is considered hyperendemic in many developing countries such as India, Bangladesh, Egypt, Mexico, and China. Hyperendemic countries carry an HEV prevalence of 25% of all non-A, non-B, acute hepatitis cases or have experienced a major waterborne outbreak of hepatitis E according to the Centers for Disease Control and Prevention [75]. HEV is considered endemic where there is a prevalence of less than 25% of all reported non-A, non-B acute hepatitis [75]. Endemic countries include much of Western Europe, the United States, New Zealand, many countries in South America, much of Asia, and the Middle East [75,76,77]. Trends throughout the world point to continued high anti-HEV seroprevalence and HEV infection likely due to increases in interest, awareness and surveillance efforts as well as increased spread among known animal reservoirs and hosts [20,75,76,77,78,79,80,81]. Seroprevalence reports vary dramatically from country to country and study to study with some studies reporting overall declines in seroprevalence over time, while other yield continued high levels of seroprevalence [80,82,83].
Prevalence of anti-HEV IgG tends to increase with age especially in men [80,84,85,86,87]. Humans and other animals excrete a considerable amount of virus early in the acute phase of HEV infection and likely contribute to maintain the cycle of endemicity [76]. The lack of a standardized serological assay further complicated the interpretation of the sero-epidemiological data. Therefore, development of an FDA-approved diagnostic assay for HEV should be a priority in the future.

5. Environmental Contamination and Waterborne Transmission

5.1 HEV Transmission from Sewage and Animal Manure Run-off

HEV is typically transmitted via fecal-oral route within an animal species, from animals to humans in infectious body fluids, and from contaminated food or water sources to humans and other animals. Inadequate disposal and treatment of sewage and contamination of drinking and irrigation water lead to the many epidemics in developing countries [2,88,89]. Increased rates of human HEV infection in Turkey and certain countries in Southeast Asia are associated with utilizing untreated river water for everyday tasks such as bathing, drinking, and disposal of waste products [90,91,92,93]. Environmental catastrophes and annual flooding are also associated with elevated HEV attack rates especially in regions where river, pond, or well water use is prevalent [10,92,93,94]. In both industrialized and developing countries, raw sewage water has been shown to contain infectious HEV strains that are closely related to the strains circulating in humans (genotypes 1 and 2) and other animals (genotypes 3 and 4) [95,96,97,98,99]. In the Netherlands, genotype 3 HEV RNA was detected in river water which likely originated from sewage [100]. Run-offs from animal facilities such as hog operations have been implicated in
human HEV infections with the detection of infectious genotype 3 HEV in the animal manure and wastewater [100,101].

Professionals working in close proximity to swine, swine manure, or sewage may become infected with HEV during occupational activities [70,100,102,103,104]. For example, swine workers in Valencia, Spain were found to be 5.4 times more likely to be positive for anti-HEV IgG than those not exposed to swine [104]. Utilizing a Bayesian model to account for imperfections in sero-assays leading to differences in the interpretation of serology results, Bouwknecht et al [103] found that approximately 11% of swine veterinarians, 6% of non-swine veterinarians, and 2% of the general population were positive for anti-HEV antibodies. Variation in assays, validity of serologic tests for determining HEV prevalence, the lack of standardized diagnostic tools, the potential for multiple routes of transmission, and incompletely understood transmission routes particularly in small defined populations lead to difficulty in assessing the exact risk factors for HEV infection [105,106]. For example, Vulcano et al [107] identified male housekeepers and specific pig breeders as carrying a higher prevalence of IgG anti-HEV seropositivity than previously identified in Italy and found a 5.5% seropositivity in subjects from Rieti in comparison to 2.5% from Rome, despite an overall lack of association with swine contact. In addition, pig farmers and the general population in Sweden were found to have 13% and 9% seropositivity respectively, which was higher than previously reported for populations in Europe (1-9%) and contributes to uncertainty in our current knowledge of transmission routes and risk factors for HEV infection [108]. Again, standardized serological and molecular diagnostic tests are in critical need for the study of HEV transmission and prevalence. During natural contact routes of transmission, HEV RNA is also detectable in the urine of infected swine, which likely contributes to the ease of spread in confined swine operations and may pose as an alternate route of exposure for
humans [109]. Contaminated water and sewage may serve as sources for HEV infection in both humans and other animals. Current research indicates the potential for transmission through these sources; however, further analysis of these sources in regards to all genotypes of HEV will better assess the overall public health risk.

5.2 Surface Water Contamination and Transmission of HEV

Surface water is easily contaminated by stable fecal-shed viruses such as HEV and acts as a public health hazard [110]. The quality of surface water directly affects populations utilizing the source since drinking water, and intensive farming practices lead to higher detection rates of viruses within these sources [110,111]. In Canada, HEV genotype 3 detected from field-grown strawberries shared 99% nucleotide sequence identity with local swine HEV strains [112,113]. In Slovenia, genotype 3 HEV was recovered from surface waters as well as from 20% of fecal samples in local pig farms [114]. Typical irrigation practices allow HEV and other enteric and hepatic viruses to impact surface water quality and elevate the potential for human exposure to pathogens [115,116]. Contaminated produce may serve as a source for autochthonous HEV cases in non-endemic regions [112,117]. In all cases of HEV detection in water or produce, the contamination levels were not assessed for further infectivity of humans or animals. The ability to recover infectious virus both from the local pig farms, the surface waters, and from produce receiving contaminated water would indicate that the virus is stable enough to be transmitted in these sources. Therefore, further infectivity studies should be done to assess the ability to transmit and cause infection especially in cases where the virus contamination levels are low.
5.3 Coastal Water Contamination and Transmission of HEV

Coastal waters may also be contaminated by HEV leading to accumulation of the virus in the digestive tissues of shellfish, which poses a risk of human infection through ingestion. Most often, mussels, cockles, and oysters are eaten raw or slightly cooked, and HEV is stable in both alkaline and acidic environments, frozen for more than 10 years, and remains infectious at up to 60°C, suggesting that a raw, rare-cooked, or slightly steamed contaminated seafood may transmit HEV to consumers [118,119]. Shellfish have been implicated in an outbreak of HEV occurring aboard a cruise ship in European waters and HEV has been identified in commercial mussels obtained from three European countries (Finland, Greece, and Spain) [120,121]. In Scotland, 92% of bivalve mussels collected were tested positive for HEV RNA with the viral sequences clustering with genotype 3 human and swine HEV [122]. Case reports of hepatitis E in England, Italy, and France reveal shellfish consumption as a common source risk factor for HEV infection [79,123,124]. In addition, genotype 3 swine HEV has been detected in shellfish in Korea and Japan [125,126,127]. Travelers to hyperendemic and endemic regions of the world are at an increased risk of acquiring HEV infection from contaminated water and seafood, but industrialized countries are not exempt [77].

6. Foodborne Transmission and Food Safety

The meat products from HEV-infected reservoir animal species are capable of transmitting HEV to humans and are a public health concern [75,76,88]. HEV primarily replicates in the liver of infected animals; however, extra-hepatic sites of HEV replication have also been demonstrated in the gastrointestinal tissues, mesenteric and hepatic lymph nodes, and spleen [20]. In addition to the liver tissues, HEV RNA has been detected from the stomach, kidney, salivary glands, tonsils,
lungs, and multiple muscle masses of pigs and chickens when inoculated intravenously [128,129,130].

Consumption of undercooked or raw organs or tissues from infected swine has been linked to numerous cases of hepatitis E worldwide. For example, three cases of hepatitis E in Japan were associated with the consumption of undercooked or raw pork presumably from the same barbeque restaurant [131]. Nine of ten clinical cases of hepatitis E from 2001 to 2002 had a history of consuming undercooked pork 2-8 weeks before the onset of clinical signs and 1.9% of pig livers tested from local groceries in Hokkaido, Japan were positive for genotype 3 or 4 HEV RNA [13]. Consumption of pig liver or intestines is considered as a risk factor for HEV infection [131]. Cases of hepatitis E in Japan were also linked to the consumption of contaminated wild boar meat [132,133,134,135]. Wild boar populations in Italy and South-eastern France had detectable levels of HEV RNA in 2.5% of liver samples and 25% of bile samples, respectively [136,137]. Boar meat consumption was positively associated with HEV infection in a case-control study in Germany [138]. Cases of acute hepatitis E associated with genotype 4 HEV have been confirmed in South Korea, presumably due to the consumption of raw wild boar bile juice [139]. Human patients with acute HEV infections in France were linked to the consumption of figatellu sausage (Corsican raw pig liver dish). The HEV sequences recovered from the figatellu products in local grocery stores were essentially indistinguishable from the viral sequences recovered from the human patients, thus providing compelling evidence for foodborne HEV transmission [11,140]. The HEV present in the pig liver sausage from manufacturers in France was shown to be infectious utilizing a 3D HEV cell-culture system [141]. Commercial pig livers tested in the United States, Germany, and the Netherlands also carried detectable levels of HEV RNA in 11%, 4%, and 6.5% of the samples tested, respectively [142,143,144]. At slaughterhouses in Bavaria, Germany, 68.6%
of the serum samples and 67.6% of meat juice samples were tested seropositive for HEV antibody, indicating animal exposures to HEV prior to slaughter [145]. In Italy, an overall 87% anti-HEV seropositivity was detected in slaughterhouse swine and 64.6% were positive for HEV RNA indicating both a high level of exposure to HEV and a similarly high level of active virus infection at the time of slaughter [146]. Similar investigations of pork production chains in the Czech Republic, Spain, and the United Kingdom revealed detectable, infectious HEV at both processing locations and point of sale [147]. Genotype 4 HEV has also been identified in a small percentage of pig livers collected from markets in India and carry a 90–91% nucleotide sequence identity with the local swine HEV isolates [148]. Other reports identify Indian strains of genotype 4 swine HEV as genetically distinct from genotype 1 human HEV strains circulating in the region further convoluting the route of transmission [149]. Human consumption of genotype 4 HEV-contaminated pork livers leading to disease has not yet been reported in India, which may be due to differing culinary habits [11,140]. It is likely that the genotype 4 swine HEV in India does cause sporadic cases of acute hepatitis E in humans through zoonotic infection, although such rare and sporadic cases of genotype 4 hepatitis E may be masked by the more prevalent and explosive form of genotype 1 hepatitis E in India.

In addition to pork, game meats such as deer have also been implicated as sources for HEV transmission to humans following the detection of near identical HEV sequences from leftover Sika deer meat and four hepatitis E patients in Japan who previously consumed the deer meat as sushi [14,20,150]. A locally caught wild deer carried a nearly identical HEV isolate that was later confirmed in local wild boar populations in Japan as well [150]. Sashimi style deer meat is usually consumed in Japan where a case-control study attributed raw deer meat as a risk factor for anti-HEV seropositivity after identifying a positive association between deer meat consumption and a
previous case of hepatitis E [14,151]. Elevated risks indicate that within this defined case-control population, those who consumed raw deer meat were more likely to be positive for HEV antibodies indicating exposure to the virus, while those who did not consume the deer meat had a lower level of exposure based on seropositivity [14,151]. Consumption of game meats including wild boar, deer, and hare was independently associated with HEV infection in organ transplant recipients in France with an odds ratio of 2.32 [152]. Combined, these studies clearly identify wild and domestic pork products and game meats as sources for human HEV infection and implicate foodborne transmission as a common route for HEV infection.

7. Known and Potential Animal Reservoirs

A number of animals are known to serve as the natural hosts and reservoirs for HEV. HEV has been genetically identified from rat, wild boar, domestic swine, mongoose, rabbits, chickens, ferrets, cutthroat trout, bats, and deer [17,18,19,23,25,26,34,61,139,153]. Anti-HEV antibodies have been detected in a number of other animal species including cattle, sheep, and goats with the potential to carry novel strains of HEV [1,154]. With the advance of modern molecular biology techniques such as metagenomics and pyrosequencing, it is expected that the host range of HEV will expand and novel strains of HEV will be identified from other animal species in the near future.

7.1 HEV in Avian Species

Avian HEV was identified as such in 2001 from chickens with Hepatitis-Splenomegaly (HS) syndrome in the United States [34]. Likewise, Big Liver and Spleen Disease virus (BLSV) in Australia presented similarly with an approximately 80% nucleotide sequence identity to avian HEV [34,73]. These two previously identified syndromes (HS and BLS) are assumed to be caused
by variant strains of the same virus, avian HEV, which now encompasses three distinct, but related genotypes worldwide [24,73,155,156]. In the United States, an estimated 71% of chicken flocks and 30% of individual chickens are positive for avian HEV [36]. Avian HEV infection in chickens is age-dependent with 17% of seropositive chickens under 18 weeks of age and 36% of seropositive adult chickens [36,157]. Avian HEV has been shown to cross species barriers and infect turkeys [71]. It is currently unknown, however, whether avian HEV is capable of transmission to humans or other mammalian species; although, rhesus monkeys and mice are not susceptible to infection by avian HEV under experimental conditions [1,73].

7.2 HEV in Domestic and Wild Swine Species

Since its discovery in domestic swine in the United States in 1997, swine HEV strains have been identified worldwide in both domestic and wild swine with widely variable prevalence [11,61]. Studies of prevalence across Japan revealed that anti-HEV antibody is present in 93% of all domestic swine farms tested and that all swine HEV isolates belong to either genotype 3 or 4 [24,48,158,159]. Prevalence of anti-HEV antibodies in wild boars in Japan is also widely variable ranging from 4.5% to 34.3% based on geographic regions with genotype 3 or 4 HEV RNA detection rates ranging from 1.1% to 13.3% [48]. In the Netherlands, domestic swine farms carried a prevalence of 55% for HEV RNA in the feces, while 86.2% and 47.1% of 18-week-old pigs in Canada shed HEV virus in feces and serum, respectively, with a declination as the pigs aged [63,160]. In Spain, the prevalence of anti-HEV antibodies on commercial swine farms reached 98%, while the anti-HEV prevalence in New Zealand, Laos and Brazil is 90%, 46% and 81%, respectively [20,161,162,163,164]. The anti-HEV seropositivity in wild boars varied from 17-50.3% with HEV RNA detected in up to 25% of samples in Germany, Italy, Spain, Australia, and Hungary [15,136,137,165,166,167]. In the United States, swine HEV infection in pig farms is
also widespread, and the majority of pigs became seropositive to HEV antibodies at approximately 3 months of age [61]. It appears that genotype 3 or 4 HEV infection in pigs is widespread in the pig population worldwide, thus raising a concern for zoonotic infection and pork safety.

7.3 HEV in Deer

Deer have been implicated both acting as animal reservoirs for HEV and acting as vehicles for human infection [12,14,20,150]. The Sika and Yezo deer in Japan carried a 3% and 35% anti-HEV seroprevalence respectively, with a positive association with HEV infection in humans and nearly identical nucleotide sequence identity with HEV strains from local wild boars [7,14,150,168]. In Hungary, the European roe deer was implicated as a reservoir species for HEV, and in the Netherlands 5% of red deer were also found positive for antibodies to HEV [165,167,169]. White-tailed deer in Northern Mexico carried a 62.7% anti-HEV seropositivity [170]. Increasing management of deer including feeding, watering, movement of groups, and fencing for hunting purposes in Mexico offers the ability for pathogens such as HEV to transfer between groups of deer and humans readily and may serve to disseminate pathogens to animals within the United States [170]. Sharing of habitats between wild boar and deer may play a role in the ability to harbor and transmit HEV to humans. However, without additional direct evidence of transmission within the deer species, it is difficult to determine whether deer acts as incidental or natural hosts to HEV infection [1,20,76].

7.4 HEV in Ruminants

Ruminant (cattle, sheep and goat) strains of HEV have yet to be uncovered; however, multiple studies of anti-HEV seroprevalence indicated the possibility of their existence [7,165]. In Egypt, 11% of cows, 14% of buffalo, 4.4% of sheep, and 9.4% of goats were tested positive for HEV.
antibodies [171]. Approximately 4.4–6.9% of cows and 0% of goats in India, 1.4% of cows and 0% of sheep and goats in Brazil were reportedly tested positive for anti-HEV antibodies [172,173]. Reports of anti-HEV seropositivity from China varied drastically from 6-93% of cattle and 10-12% of sheep [174,175,176,177]. A short sequence (189 bp) of a genotype 4 HEV has been reportedly identified in bovid species, although independent confirmation of this unsubstantiated report is still lacking [1,7,76].

Despite the abundant serological evidence for an HEV-related agent in ruminants, definitive genetic identification of HEV from ruminants is still lacking. It is possible that the strain carried by ruminants is very divergent genetically from the known HEV strains thus leading to failure to genetically identify the virus based upon current techniques. The serological data from ruminants is based upon cross-reaction of the ruminant serum samples with known HEV proteins such as ORF2 [7,165,171,172]. The validity of such serological data has been questioned due to the fact that the assays may not be specific, they do not identify the actual virus, and they may allow cross-reactivity with non-viral proteins that share a certain level of sequence homology. Research in this area must continue to better address these concerns and confirm the source of anti-HEV seropositivity in ruminants. Given the wide use of cattle, sheep, and goats in the human food chain, the genetic identification of these ruminant strains of HEV would be of a potential public health concern.

7.5 HEV in Rats

The rat strain of HEV was identified in wild Norway rats from Hamburg, Germany with 59.9% and 49.9% nucleotide sequence identity with known human and avian HEV strains, respectively [18]. Rats in the United States, Germany, Indonesia, China, and Japan are also tested seropositive for HEV antibodies in several studies with variable prevalence [18,178,179,180,181].
Overall, 44% of rats in Louisiana, 77% in Maryland, 90% in Hawaii, 59.7% of rats of the genus *Rattus* from across the United States, 32% of Norway rats in Japan, and 13% of black rats in Japan were tested positive for antibodies to HEV [178,179,182,183]. Most recently in China, 23.3% of rats were positive for anti-HEV IgG with the highest prevalence of 45.3% from rats caught at garbage dump sites [180]. In Indonesia, 18.1% of rats were tested positive for anti-HEV antibodies and 14.7% positive for HEV RNA [181]. Recently, genotype 3 rat HEV strains have been genetically detected from wild rats in the United States, suggesting the potential for zoonotic transmission and the genetic variability of rat HEV [1,182]. Further studies are warranted to independently confirm the existence of genotype 3 HEV in rats, especially since, under experimental condition, laboratory rats are not susceptible to experimental infection by genotype 3 HEV [184].

7.6 HEV in Rabbits

Rabbits may serve as reservoir hosts for HEV transmission to humans given the genetic identification of zoonotic genotype 3 strains of HEV from rabbits in China, the United States, and France [17,153,174,185]. Rabbits are susceptible to experimental infection by genotype 4 human HEV, and the infected rabbits developed viremia, seroconversion to anti-HEV, and fecal virus shedding [153,185]. The rabbit HEV is genetically and antigenically closely related to other mammalian HEV. The capsid protein of the genotype 3 rabbit strain of HEV was capable of cross-reacting with antibodies from other strains of HEV including rat, swine, human, and chicken [1,185,186]. The prevalence of HEV antibodies in farmed rabbits is reportedly 57% in the Gansu province in China, 54.6% in Beijing, China, and 36.5% in two rabbit farms in Virginia, USA, while HEV RNA has been identified in 7.5%, 7.0%, 16.5%, and 15.3% of the rabbits, respectively [17,153,174]. In France, HEV RNA was also identified from 7.0% of farmed rabbits,
while 23.0% of wild rabbits were also positive for HEV RNA [185]. It appears that rabbits could be an important reservoir for HEV infection in humans, and in-depth studies of its ability to infect across species barriers and associated zoonotic risks in the future are needed.

### 7.7 HEV in Other Species

Other known animal strains of HEV genetically identified thus far include mongoose, ferret, bat, and fish [1,23,25,26,187,188]. Wild mongoose in Okinawa, Japan carried genotype 3 HEV strains and the prevalence of anti-HEV seropositivity varied from 8 to 21% [187,188]. In the Netherlands, ferrets carried a strain of HEV that shared a 72.3% nucleotide sequence identity with that of the rat HEV [23]. The cutthroat trout in the United States also carried a unique strain of HEV with only 13 to 27% sequence identity with known mammalian and avian HEV strains [26]. The zoonotic potentials of these novel animal strains of HEV are not altogether understood, but the ever-expanding host range and high levels of anti-HEV seropositivity among mammalian species suggests transmission is common and thus may pose a potential public health concern.

### 8. Animal Handling and Zoonotic Transmission

Contact exposure to infected animals leads to an elevated risk for HEV transmission in humans. Swine veterinarians in the United States were shown to have a 27% seropositivity to genotype 3 swine HEV in comparison to 16% of the normal blood donors [189]. Individuals from states in which swine production plays a key role were more likely to be seropositive to HEV than other non-major swine states [189]. Incidents such as needle sticks while working with swine were found to be 1.9 times more likely positive for HEV antibodies in swine veterinarians [189]. Pig handlers such as veterinarians, breeders, and farmers in China, Thailand, the Netherlands, Sweden, Moldova, and the United States were also more likely seropositive to swine
HEV [103,108,190,191,192]. In Sweden, 13% of pig breeders were positive for antibodies to HEV [190]. In the Netherlands, 11% of swine veterinarians were positive in comparison to 6% of non-swine veterinarians and 2% of the general population [108]. In North Carolina, swine handlers carried a 4.5 times higher rate of seropositivity in comparison to non-swine workers [191]. In Moldova, 51% of swine farmers were positive in comparison to 25% of non-swine occupations [103]. Taken together, swine are a major reservoir for HEV and occupational contact with infected swine is a risk factor for zoonotic HEV transmission in humans.

Contact with swine is the most widely recognized route for occupational exposure to HEV; however, the multitude of novel strains of HEV in wildlife and other domestic animal species suggest additional mechanisms of transmission. For example, field workers at the Iowa Department of Natural Resources who work with a variety of wildlife species had a higher prevalence for HEV antibodies in comparison to normal blood donors [193]. While exposure to HEV, identified by the presence of anti-HEV antibodies in these populations does not in itself indicate a disease, it does identify a route of transmission and exposure that should be further assessed and acknowledged as a preventive measure against this important disease. Examination of these additional mechanisms is vital to understanding the full-spectrum of public health risk associated with HEV infection.

9. Conclusions

The zoonotic risk of HEV is well established; however, the ever-expanding host range and identification of new animal reservoir species poses a significant public health concern. Seroprevalence in human and other animal species varies drastically between studies and countries with no clear understanding of the overall problem, and this is largely due to the lack of an established FDA-approved serological diagnostic assay. Numerous animal species were tested
seropositive for IgG anti-HEV, although HEV was not genetically identified from all seropositive animal species. Detection of HEV in sewage, water sources, coastal and surface waters, and produce poses environmental safety concerns even in industrialized countries where waterborne origins of human hepatitis E cases were previously considered rare. Foodborne cases of hepatitis E in humans are increasingly common and likely underestimated in the medical community. Sporadic and cluster cases of hepatitis E occur after consumption of undercooked or raw animal meats. Prevention of foodborne HEV transmission relies on avoiding consumption of undercooked animal meats especially when immunocompromised, following good hygiene practices, and being aware of increased risks when traveling to endemic or hyperendemic regions of the world. Despite the clear risk, prevention strategies are currently minimally implemented. A vaccine against HEV has recently become available in China but not in other countries. Surveillance, vaccination, decontamination of sewage and water sources, and public education will help prevent current and future endemics or epidemics lowering the human burden. The development of a vaccine against the zoonotic swine HEV would reduce foodborne and swine contact cases in humans as well as diminish the spread of the virus between animal species. Control of animal waste, run-off, and decontaminated sewage is key to limiting the spread of HEV to coastal and surface waters and in turn reducing concomitant contamination of shellfish.

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This review article encompasses a comprehensive literature review with a focus on food and environmental safety and zoonotic risk of HEV. Due to the narrow scope of the topic and space
constraints, many important articles regarding HEV may be unintentionally excluded from this review. We have attempted to include the most recent publications including review articles in order to provide the reader with up to date and comprehensive information on the topic.

**Conflict of Interest**

The authors declare no conflict of interest.


Chapter II: Naturally Occurring Animal Models of Human Hepatitis E Virus Infection

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Abstract

Hepatitis E virus (HEV) is a single-stranded, positive-sense RNA virus in the family *Hepeviridae*. Hepatitis E caused by HEV is a clinically important global disease. There are currently four well-characterized genotypes of HEV in mammalian species, although numerous novel strains of HEV likely belonging to either new genotypes or species have recently been identified from several other animal species. HEV genotypes 1 and 2 are limited to infection in humans, while genotypes 3 and 4 infect an expanding host range of animal species, and are zoonotic to humans. Historical animal models include various species of non-human primates which have been indispensable for the discovery of human HEV and for understanding its pathogenesis and course of infection. With the genetic identification and characterization of animal strains of HEV, a number of naturally-occurring animal models such as swine, chicken and rabbit have recently been developed for various aspects of HEV research including vaccine trials, pathogenicity, cross-species infection, mechanism of virus replication, and molecular biology studies. Unfortunately, the current available animal models for HEV are still inadequate for certain aspects of HEV research. For instances, an animal model is still lacking to study the underlying mechanism of severe and fulminant hepatitis E during pregnancy. Also, an animal model that can mimic chronic HEV infection is critically needed to study the mechanism leading to chronicity in immunocompromised individuals. Genetic identification of additional novel animal strains of HEV may lead to the development of better naturally-occurring animal models for HEV. This article reviews the current understanding of animal models of HEV infection in both the natural and experimental infection setting and identifies key research needs and limitations.

**Keywords:** Hepatitis E virus (HEV); Animal models; Cross-species infection; Rabbit; Swine; Chicken
Introduction

Hepatitis E virus (HEV)

HEV is classified in the genus *Hepevirus* of the family *Hepeviridae* (Meng et al. 2012). The mammalian HEV is comprised of four recognized genotypes and at least two putative genotypes that are distinct in geographic locale as well as host. Genotype 1 HEV causes the majority of outbreaks of hepatitis E in humans in Asia. Genotype 2 HEV includes one Mexican strain and multiple African strains and causes large outbreaks in humans. In addition to humans, genotype 3 HEV has been isolated from a wide variety of animal species including wild and domestic swine (de Deus et al. 2008; Meng et al. 1997; Takahashi et al. 2004), deer (Takahashi et al. 2004), mongoose (Nakamura et al. 2006), rats (Lack et al. 2012), and rabbits (Cossaboom et al. 2011; Zhao et al. 2009) and is associated with zoonotic transmission causing sporadic, cluster, and chronic cases of hepatitis E in humans (Meng 2010). Genotype 4 HEV infects humans as well as wild and domestic swine with zoonotic transmission to humans causing sporadic cases of hepatitis E (Meng 2013). (Table 1)

In addition to the zoonotic genotypes 3 and 4 HEV strains mentioned above, genetically divergent strains of HEV have also been identified from several other animal species including chicken (Haqshenas et al. 2001; Marek et al. 2010; Payne et al. 1999), bat (Drexler et al. 2012), fish (Batts et al. 2011), rat (Johne et al. 2011), ferret (Raj et al. 2012), and wild boar (Takahashi et al. 2011). Additionally, antibodies to HEV have been reportedly detected from horses (Saad et al. 2007) and ruminant species including cattle, sheep, and goats (Sanford et al. 2012a); although the source of seropositivity in these species remains unknown (Meng 2013). The animal host range of HEV has expanded dramatically over the past decade from the initial identification of HEV in swine in 1997 (Meng et al. 1997) and chickens in 2001 (Haqshenas et al. 2001), to a multitude of
animal species acting as both reservoir and host more recently. Further research will likely expand the host range of the virus to include additional novel strains from many more other animal species. Reclassification and nomenclature changes for HEV are eminent with the recent identification of several novel strains (Table 1).

Hepatitis E

HEV is transmitted via fecal-oral route through contaminated food or water and typically causes an acute icteric disease known as hepatitis E (Meng 2010; Purcell 2001). The majority of patients experience an asymptomatic course of disease in which the virus is quickly cleared with no major complication (Meng 2010). Symptomatic patients experience a range of symptoms including anorexia, jaundice, darkened urine coloration, hepatomegaly, myalgia, elevated ALT levels in the blood, and occasionally abdominal pain, nausea, vomiting, and fever (Purcell 2001). Acute HEV infection in humans begins with a typical incubation period of 2 weeks to 2 months, a transient viremia period with viral shedding in the feces, a symptomatic phase lasting days to weeks, and jaundice apparent 2-3 weeks into the course of infection (Purcell 2001). The severity of HEV infection is considered dose-dependent with alcohol use or other concurrent hepatic diseases as contributing factors (Purcell 2001). Immunocompromised individuals infected with HEV such as organ transplant recipients are at a high risk of developing chronic hepatitis E (Kamar et al. 2013; Kamar et al. 2008). Pregnancy associated complications with concurrent HEV infection include death of both the mother and fetus, abortion, premature birth, and death of the baby shortly after birth with no known mechanism for the severe hepatitis E manifestation (Navaneethan et al. 2008). The mortality rate ranges from 0.5-4% in immune competent
individuals and concurrent pregnancy attributed to increases in mortality up to 20% (Aggarwal 2011).

Hepatitis E affects humans in both industrialized and developing countries worldwide. Industrialized countries experience sporadic and cluster cases of hepatitis E associated with ingestion of contaminated animal meats, shellfish, and contact with infected animals (Meng 2013; Teo 2010). Developing countries such as Bangladesh, Egypt, Mexico, China, India, and parts of Africa experience large waterborne outbreaks due to poor sanitation conditions and a hyperendemic status in the population (Teo 2010). Genotypes 1 and 2 HEV strains are limited to the human population, while genotypes 3 and 4 strains are zoonotic and infect humans and other animals. Human to human transmission of HEV is considered rare; however, transmission through blood products by transfusion has been reported (Pavio et al. 2010).

Animal models of human hepatitis E virus

Several animal species serve as useful models for human HEV due to their susceptibility to infection by human HEV strains (Table 2). Cynomolgus and rhesus monkeys are susceptible to genotypes 1-4 HEV and serve as the primary model for genotypes 1 and 2 human HEV strains (Meng 2010; Purcell and Emerson 2001). Swine serve as a reservoir for genotypes 3 and 4 human HEV and a natural animal host for HEV infection (Halbur et al. 2001). Despite production of efficient virus replication, both the non-human primate and swine models have limitations in reproducing clinical aspects of hepatitis with minimal elevations in serum levels of liver enzymes and moderate pathological liver lesions present (Halbur et al. 2001; Meng 2010). Rabbit HEV recently identified in China (Zhao et al. 2009), the United States (Cossaboom et al. 2011), and France (Izopet et al. 2012) likely serves as a useful model of genotype 3 human HEV infection.
with successful transmission of the virus to cynomolgus monkeys (Liu et al. 2013) and pigs (Cossaboom et al. 2012) thereby demonstrating the potential for cross-species infection (Table 2).

**Historical Animal Models for Human Hepatitis E**

In 1983, an experimental transmission study to a human volunteer as well as cynomolgus monkeys identified virus-like-particles (VLP) in stool by immune electron microscopy that became what is now HEV (Balayan et al. 1983). After ingestion of the stool samples collected from Afghan patients with signs consistent with viral hepatitis, the human volunteer developed clinical symptoms consistent with acute viral hepatitis with a non-A, non-B hepatitis etiology and VLPs were identified in the volunteer’s stool samples (Balayan et al. 1983). In addition, cynomolgus macaques inoculated with fecal samples containing the VLPs responded similarly with elevations of serum liver enzymes, histopathologic liver lesions, excretion of VLPs in feces, and antibody responses with confirmed reaction to the VLPs (Bradley et al. 1987). Anti-viral antibodies were confirmed in serum samples obtained from patients involved in outbreaks of non-A, non-B, acute hepatitis in various other countries, confirming an association between the identified virus and human infections (Khuroo 2011; Purcell and Emerson 2001).

Non-human primates such as rhesus macaque and chimpanzee have served as important animal models for HEV. Following the initial infection of cynomolgus monkeys with fecal samples containing VLPs, various species of non-human primates were utilized in an attempt to better characterize the then unknown virus (Balayan et al. 1983; Bradley et al. 1987). Macaques developed clinical signs consistent with acute viral hepatitis, occasionally excreted the VLPs in feces, and developed antiviral antibodies (Arankalle et al. 1995; Tsarev et al. 1993a). Chimpanzees and tamarins were inconsistently infected in these initial attempts (Arankalle et al. 1988; Bradley
et al. 1987; McCaustland et al. 2000). Subsequent attempts to infect other non-human primate species have yielded mixed results with tamarins occasionally developing infection, but chimpanzees (Arankalle et al. 1988), pig-tailed macaques (Tsarev et al. 1993b), vervets (Tsarev et al. 1993b), owl monkeys (Ticehurst et al. 1992), squirrel monkeys (Tsarev et al. 1993b), and patas monkeys were all susceptible through experimental infection. Rhesus and cynomolgus monkeys both in captivity and wild-caught had serological evidence of natural exposure to HEV and the seroprevalence was age-dependent with the majority positive for anti-HEV antibodies at 1 year and older for both species (Arankalle et al. 1994; Purcell and Emerson 2001; Tsarev et al. 1993a). Based on the transmission studies, chimpanzees (Arankalle et al. 1988), rhesus (Arankalle et al. 1995), and cynomolgus monkeys (Bradley et al. 1988; Tsarev et al. 1993a) were the most susceptible to both human strains of HEV (genotypes 1-4) and animal strains of HEV (genotypes 3 and 4) and are considered suitable models for HEV studies (Purcell and Emerson 2001) (Table 2). However, the restricted procedures, limited animal resources and ethical concerns have limited the widespread use of these historical non-human primate models in HEV research today.

Naturally Occurring HEV Infections in Animals

Known animal strains of HEV

The ever-expanding host range for HEV currently includes a variety of animal species that serve as both reservoirs for human infections as well as hosts of genetically diverse but related viruses (Table 1). The most well-characterized animal strains of HEV include genotypes 3 and 4 swine HEV from domestic and wild pigs (Meng et al. 1997), and avian HEV genotypes 1-3 (Haqshenas et al. 2001; Huang et al. 2002). Rabbit HEV has recently been identified and genetically characterized as a genotype 3 from rabbits in China (Zhao et al. 2009), the United
States (Cossaboom et al. 2011), and France (Izopet et al. 2012). Rats (Johne et al. 2011) and ferrets (Raj et al. 2012) each carry HEV-related strains genetically distinct from other mammalian and avian HEV and cluster together as a new putative genus proposed as Orthohepevirus (Meng 2013). Cutthroat trout virus (Batts et al. 2011) resembles mammalian HEV in its genomic organization despite low nucleotide sequence identity and likely represents a putative genus proposed as Piscihepevirus (Meng 2013). Genotype 3 HEV has also been isolated from wild mongooses in Japan (Nakamura et al. 2006). Recently, a novel phylogenetic clade of HEV obtained from Western African, Central American, and European bat species was identified, although evidence for transmission from bats to humans was lacking (Drexler et al. 2012). Deer have been implicated in foodborne transmission of genotype 3 HEV to humans and share high nucleotide sequence identity to genotype 3 wild boar HEV in Japan (Takahashi et al. 2004). The genetic identification of these diverse animal strains of HEV provided opportunities for developing new and useful naturally-occurring animal models for HEV in the future.

Animal species with only serological evidence of HEV infection

The presence of anti-HEV antibodies in serum indicates the exposure to and potential infection by HEV. Ruminant species including goats (Sanford et al. 2012a), cattle, and sheep have been reported as seropositive to anti-HEV antibodies without definitive genetic identification of HEV (Meng 2013). Horses were reportedly a potential reservoir of HEV by the presence of anti-HEV antibodies and viral RNA; however, the close-relatedness of virus from horses to human HEV strains in Cairo (97-100% nucleotide sequence identity) raises questions about the authenticity of these sequences as true virus from horses (Saad et al. 2007). Anti-HEV antibodies have also been reportedly detected in both dogs and cats with no detection of HEV-related
sequences (Pavio et al. 2010). Definitive genetic identification of the sources for anti-HEV seropositivity in these animal species will discover new animal strains of HEV, and thus leading to the potential development of additional naturally-occurring animal models for HEV in the future.

Naturally Occurring Animal Models for Human Hepatitis E

Swine model

*Discovery and prevalence of swine HEV in pigs.* Discovered from pigs in the United States in 1997, swine HEV became the first known animal strain of HEV (Meng et al. 1997) and has since been identified in domestic and wild swine worldwide. Swine HEV was initially discovered via identification of anti-HEV seropositive adult pigs followed by a prospective study on piglets from a herd in Illinois that lead to the recovery of a novel virus (Meng et al. 1997). The novel virus was successfully transmitted to specific-pathogen-free (SPF) pigs and the same virus was recovered from the experimentally infected SPF pigs, thereby satisfying Koch’s postulates (Meng 2010).

Swine serve as a major reservoir for zoonotic genotypes 3 and 4 HEV (Meng 2010). Serological and molecular prevalence studies of swine HEV yield widely variable results in both domestic and wild swine species in essentially all swine-producing countries. In the United States, the majority of pigs develop seropositivity to HEV by 3 months of age with dispersion of the virus in most herds (Meng et al. 1997). In Canada (Leblanc et al. 2007), Japan (Takahashi and Okamoto 2013), many European countries (Pavio et al. 2010; Rutjes et al. 2009), and China (Meng et al. 1999), swine HEV infection is highly prevalent in both domestic and wild swine species irrespective of the human population. Swine HEV is equally dispersed in developing and industrialized countries worldwide.
Pathogenesis and course of infection of swine HEV in the pig model. Following the subclinical course of HEV infection, swine develop only mild microscopic lesions in the liver and associated lymph nodes (Meng et al. 1997). Microscopic lesions include mild to moderate multifocal and periportal lymphoplasmacytic hepatitis and mild focal hepatocellular necrosis (Halbur et al. 2001). A prospective study of four piglets naturally infected by swine HEV identified no apparent gross lesions in 19 different tissues during necropsy, but characteristic microscopic lesions of hepatitis and lymphoplasmacytic enteritis in all as well as multifocal lymphoplasmacytic interstitial nephritis in three of the four (Meng et al. 1997). Under experimental conditions, pigs infected with swine HEV developed no clinical abnormalities, but were consistent in microscopic liver lesions as the naturally infected pigs, and HEV RNA was detectable in feces, liver tissues, and bile (Halbur et al. 2001). Pathological lesions in wild boars have not been investigated; however, the similarity between domestic and wild swine strains leads to speculation that clinical and pathologic effects are likely similar.

Domestic pigs are typically infected by HEV at 2-4 months of age with a transient viremia lasting 1-2 weeks and fecal viral shedding lasting 3-7 weeks (de Deus et al. 2008). By 18 weeks of age, up to 86% of pigs are naturally infected (Leblanc et al. 2007). The source of infection is thought to be virus shed in large amounts in feces with transmission via fecal-oral route most common (Meng 2011). Naïve pigs acquire and spread the virus through direct contact between pigs as well as fecally-contaminated feed and water sources in their environment. Following the waning of maternal antibodies around 8 weeks of age, piglets become infected by swine HEV and seroconvert first with IgM anti-HEV antibodies that peak in conjunction with peak fecal viral shedding followed by seroconversion of IgG anti-HEV antibodies with subsequent clearance of
the virus from the feces (de Deus et al. 2008; Leblanc et al. 2007). Genotypes 3 and 4 HEV infections carry a subclinical course in both naturally and experimentally infected swine with no observable clinical disease or elevation in liver enzymes (Halbur et al. 2001). Experimentally, swine are readily infected via intravenous inoculation; however, oral route of inoculation is inefficient (Meng 2011).

Wild boars are assumed to be a natural reservoir for HEV as well due to the recovery of nearly genetically identical strains of HEV in deer cohabiting forestry land in Japan (Takahashi et al. 2004). As a model for human HEV infections, swine efficiently produce infection with genotypes 3 and 4 HEV and act as the main reservoir for foodborne and zoonotic HEV transmission to humans. Therefore, this naturally-occurring swine model is very useful for the study of various aspects of HEV replication, pathogenesis and cross-species infection (Meng 2010) (Table 2). The major drawback of the naturally-occurring swine HEV model is that it does not reproduce a hepatic disease with overt clinical signs, thus limiting its usefulness in pathogenicity studies.

Chicken model

*Discovery and prevalence of avian HEV in chickens.* Avian HEV was identified in the United States in 2001 from chickens with Hepatitis-Splenomegaly syndrome (HSS) (Haqshenas et al. 2001). Big Liver and Spleen Disease Virus (BLSV) presented similarly in chickens in Australia with approximately 80% nucleotide sequence identity to avian HEV (Marek et al. 2010; Payne et al. 1999). These two syndromes (HSS and BLS) are now known to be caused by variants of the same virus within the avian HEV clade. Avian HEV currently consists of at least three genotypes
(1-3) throughout the world and shares approximately 60% nucleotide sequence identity with human HEV strains (Marek et al. 2010), but is not known to infect humans.

Avian HEV infection in chickens affects approximately 71% of chicken flocks and 30% of individuals overall within the United States (Huang et al. 2002). Avian HEV transmits most likely via fecal-oral route and spreads easily between and within chicken flocks (Meng et al. 2008). The infection in chickens is age-dependent affecting 17% of chickens under 18 weeks of age but 36% of adults were positive for anti-HEV antibodies (Huang et al. 2002).

*Pathogenesis and course of infection of avian HEV in the chicken model.* Following avian HEV infection, few birds show clinical signs prior to death. Post-mortem evaluations reveal regressive ovaries, serosanguinous abdominal fluid, enlarged, hemorrhagic, and necrotic livers, and enlarged spleens (Meng et al. 2008). Microscopic evaluations identify inflammatory cellular infiltrations within the liver parenchyma (Billam et al. 2005). Likewise, experimentally infected birds consistently present with lymphocytic periphlebitis and phlebitis in the liver at microscopic evaluation with enlarged and hemorrhagic livers in approximately 25% of the infected birds at gross evaluation (Meng et al. 2008). Avian HEV has been shown to successfully cross species barrier and infect turkeys (Sun et al. 2004); however, attempts to experimentally infect rhesus macaques (Huang et al. 2004) and mice were unsuccessful (Meng et al. 2008).

Avian HEV genotypes 1-3 correspond with mortality rates in chickens ranging from 0.3-1.0% of the overall flock and a high level of subclinical infection. Typical clinical signs include egg drop, hepato-splenomegaly, and acute death of birds. In flocks displaying signs of avian HEV infection, up to 20% of hens present egg drop significantly reducing production (Meng et al. 2008). In an age-dependent fashion, broiler breeders and laying hens of 30-72 weeks of age display the
highest level of mortality (Huang et al. 2002). As a model for HEV infection in humans, avian HEV genotypes 1-3 are far more limited in their host range, but this naturally-occurring chicken model offers a unique hepatic disease model (HSS) that can be used to study at least some aspects of human hepatitis E disease (Table 2).

Rabbit model

*Discovery and prevalence of rabbit HEV in rabbits.* Farmed rabbits from the Gansu Province in China were tested positive for anti-HEV antibodies and full-length genomic sequences of HEV were determined and found to be most closely related to the zoonotic genotype 3 HEV (Zhao et al. 2009). Subsequently, studies on farmed and wild rabbits in the United States (Cossaboom et al. 2011) and France (Izopet et al. 2012) confirmed the presence of rabbit HEV related to genotype 3.

Anti-HEV antibody presence in rabbits is highly prevalent with 57%, 54.6%, and 34.6% of farmed rabbits in the Gansu province of China, Beijing, and Virginia, USA testing positive respectively (Cossaboom et al. 2011; Zhao et al. 2009). The detection of rabbit HEV RNA in fecal and serum samples also indicate a widespread infection of the virus with 7.5%, 7.0%, and 15.9% of respective farmed rabbits positive (Cossaboom et al. 2011; Zhao et al. 2009). A study in France identified a similar proportion of farmed rabbits positive for HEV RNA at 7.0%, while 23.0% of wild rabbits were also positive (Izopet et al. 2012).

*Pathogenesis and course of infection of rabbit HEV in the rabbit model.* The rabbit likely acts as a reservoir for HEV since the rabbit HEV belongs to the zoonotic genotype 3 that infects humans. The close genetic and antigenic relationship to other mammalian HEV strains indicates the potential of rabbit HEV infection in rabbits to serve as a useful naturally-occurring animal model
for human HEV study. Experimentally, rabbits are susceptible to infection by human HEV genotype 4, and rabbit HEV has been successfully transmitted to pigs (Cossaboom et al. 2012) and cynomolgus macaques (Liu et al. 2013). Anti-HEV antisera from rat, swine, human, and chicken strains of HEV cross-react with the rabbit HEV capsid protein (Cossaboom et al. 2012). Experimental infection studies in rabbits indicate the ability to produce local hepatocellular necrosis on microscopic evaluation; however, rabbits respond subclinically to experimental infection with HEV with little to no overt signs of disease. Following experimental infection, rabbits shed virus in their feces, seroconvert, and show elevations in serum alanine aminotransferase levels, indicating acute liver damage (Ma et al. 2010). More in depth studies on pathogenicity and cross-species infections are warranted to further characterize the usefulness of rabbit HEV as a naturally-occurring model for human HEV.

Other potential naturally-occurring animal models of hepatitis E

*Rat model.* A rat strain of HEV was identified in Hamburg, Germany in 2009 from Norway rats collected in sewers (Johne et al. 2011). The strain shared 59.9% and 49.9% nucleotide sequence identity with human and avian HEV strains indicating a putative new mammalian genotype. Studies from Japan, China, Indonesia, the United States, and Germany also indicate the presence of a rat strain of HEV based upon the presence of anti-HEV antibodies, and in addition to Germany, rat HEV has been genetically identified from rats in a number of other countries including the United States (Purcell et al. 2011) and Japan. In the United States, a variable prevalence of anti-HEV antibodies in rats of the genus *Rattus* exists ranging from 44%-90% in different states, and in addition to the rat HEV (Purcell et al., 2011), a genotype 3 HEV RNA has been reportedly
detected in wild rats in the United States (Lack et al. 2012), although independent confirmation of this report is still lacking.

Naturally infected rats had no overt signs of illness related to HEV infection. In experimental studies on laboratory rats infected with rat HEV, seroconversion and fecal shedding of virus were detected; however, no clinical signs were apparent (Li et al. 2013). Histopathologic evaluation of hepatic tissues from the infected laboratory rats identified mild portal inflammation, parenchymal foci of necrosis, and aggregates of lymphocytes and Kupffer cells within the lobules indicating evidence of mild hepatitis consistent with acute HEV infection (Purcell et al. 2011). As a potential model for human HEV infection, inoculation of rats with mammalian HEV genotypes 1, 2, and 4 failed to produce an efficient infection and rat HEV failed to infect rhesus monkeys (Purcell et al. 2011) (Table 2). In a separate report, Wistar rats were not susceptible to experimental infection by genotypes 1, 3, or 4 HEV, while rat HEV elicited evidence of infection as expected (Li et al. 2013). Additionally, both swine HEV and avian HEV also failed to elicit a productive infection in rats further demonstrating the limited utility of rats as a naturally-occurring model or an experimental model of human HEV infection (Krawczynski et al. 2011; Purcell et al. 2011).

Ferret. A ferret strain of HEV was genetically identified in the Netherlands in 2010. Phylogenetic analysis revealed its clustering with the rat HEV (Raj et al. 2012). Nucleotide sequence identity with known genotypes 1-4, rabbit, and avian strains of HEV ranged from 54.5% to 60.5% with the highest sequence identity to rat HEV at 72.3% (Raj et al. 2012). Little is known about the ferret HEV and the current knowledge relies on one set of samples obtained from household pets with no known illness (Raj et al. 2012). Whether ferret HEV can serve as a useful model for HEV is
unclear, however, given the close genetic relatedness of the ferret HEV to rat HEV, the usefulness of ferret HEV as a naturally-occurring animal model for HEV is likely limited.

Application of Naturally Occurring Animal Models for HEV Studies

Vaccine studies

The mouse models have been used for identification of immunogenic properties of HEV antigen and preliminary immunization trials in HEV vaccine development (Table 3). However, the mice species were non-permissive for HEV infection and were unable to be used for HEV challenge studies (Krawczynski et al. 2011). Therefore, the mouse models are mainly used for preliminary assessment of HEV vaccine antigen immunogenicity studies. HEV DNA vaccine constructs, purified VLPs, and recombinant subunit capsid protein all lead to the development of immune responses in the mouse model (Krawczynski et al. 2011).

Rhesus and cynomolgus monkeys were utilized in the vaccine preclinical and challenge studies to identify potential candidate vaccines that elicit protective immunity against known HEV genotypes (Krawczynski et al. 2011). The HEV capsid-based recombinant vaccine candidates elicit protective immune responses. A recombinant vaccine proved to be efficacious in phase II clinical trials in young men with 95% protection against HEV genotype 1 in Nepal (Shrestha et al. 2007). Another recombinant vaccine proved to be efficacious in phase II and III clinical trials in the general population (ages 16-65 years) and in pregnant women for human HEV genotypes 1 and 4 with 100% protection (Zhu et al. 2010). Efficacy was achieved with both 2 and 3-dose regimens with no serious adverse events and minimal side effects (Shrestha et al. 2007; Zhu et al. 2010). Rhesus monkeys immunized with the candidate vaccines were subsequently challenged by intravenous inoculation of human HEV genotypes 1, 2 and 3 for evaluation of protective immunity.
(Krawczynski et al. 2011). In both cases, the macaques were protected against homologous and heterologous challenge by HEV strains (Krawczynski et al. 2011). The large-scale clinical trial of a capsid-based recombinant vaccine involving 11,165 individuals (Zhu et al. 2010) has led to the approval of the first commercial HEV vaccine in China (Proffitt 2012).

Pigs have been used as a model for HEV vaccine trials and assessment of cross-protective potentials of recombinant HEV antigens, which is essential for the development of vaccines that protect against the zoonotic genotypes 3 and 4 strains of HEV. Pigs vaccinated with truncated recombinant capsid antigens derived from three different animal strains of HEV specifically induce strong anti-HEV IgG responses, and these responses are partially cross-protective against a genotype 3 mammalian HEV (Sanford et al. 2012b). In addition, prior infection of pigs with a genotype 3 swine HEV induces protective immunity enabling resistance against challenge by heterologous and homologous strains of genotypes 3 and 4 HEV (Sanford et al. 2011), further demonstrating that swine are a good naturally-occurring animal model for HEV vaccine research.

Chicken has also been used as a model for HEV vaccine studies. Immunization of chickens with avian HEV capsid protein induces protective immunity against avian HEV challenge (Guo et al. 2007), thus confirming the role of HEV capsid protein in eliciting protective immunity. The identification of B-cell epitopes within the avian HEV capsid protein that are unique to avian, swine, or human strains of HEV are useful for future diagnostic immunoassays as well as vaccine design (Guo et al. 2006). In chickens immunized with KLH (keyhole limpet hemocyanin)-conjugated capsid peptides, protection against avian HEV challenge was not achieved. In contrast, in chickens immunized with recombinant avian HEV capsid antigen, complete protective immunity against avian HEV challenge was obtained, indicating that the immunodominant epitopes in avian HEV capsid are not protective (Guo et al. 2008). Rabbits may also be useful for
HEV vaccine challenge and efficacy trials with further characterization of the course of infection and disease (Cheng et al. 2012).

Pathogenesis studies

Historically, primates served as the main animal model for HEV pathogenicity studies; however, due to ethical concerns, availability of animals, restrictions on their use, and difficulty in assessing clinical relevance since primates are not the natural host for HEV, additional naturally-occurring animal models have recently been used for pathogenicity studies (Billam et al. 2005; Purcell and Emerson 2001) (Table 3). Rhesus (Arankalle et al. 1995) and cynomolgus monkeys (Aggarwal et al. 2001; Bradley et al. 1987; Tsarev et al. 1993a) have been widely used to study HEV infection and pathogenesis; however, differences in liver enzyme elevations, virus excretion, serologic, and histopathologic results between species and in relation to a human host exist. The chimpanzee model has also been useful in analyzing the course of human infections and pathogenicity with genotype 1 and 2 human HEV, and host gene responses to HEV; although, the use of chimpanzees in HEV study is currently less frequent and more restricted (Arankalle et al. 1988; McCaustland et al. 2000). The mechanisms leading to a chronic course of HEV infection in immunocompromised individuals as well as elevated mortality rates in pregnant women of up to 25% are largely unknown due to the inability to identify an appropriate animal model (Meng 2013). Inoculation of pregnant rhesus monkeys with genotype 1 HEV failed to identify a difference with non-pregnant monkeys and was unsuccessful in reproducing the elevated mortality rates seen in pregnant women or the development of the reported severe and fulminant hepatitis E (Tsarev et al. 1995).
With the discovery of swine HEV, domestic swine became a potential model for HEV pathogenicity study; although such studies are limited due to the fact that swine infected by swine or human HEV develop only subclinical infection with mild-to-moderate pathologic lesions of hepatitis (Meng 2003; Meng et al. 1997). Experimental infections of pigs with HEV are also limited by the inability to produce a natural course of infection via natural oral route of inoculation. Even using high titers of infectious HEV stocks, many experiments have failed to initiate a productive infection via the oral route in swine (Kasorndorkbua et al. 2004). However, intravenous route of inoculation of swine HEV and human HEV in the pig model produced characteristic pathological liver lesions; although, clinical sign of hepatitis is lacking (Halbur et al. 2001). To assess the enhanced pathogenic effect of HEV infection during pregnancy observed in humans, pregnant gilts were inoculated with swine HEV (Kasorndorkbua et al. 2003). While the gilts developed active HEV infection, the offspring remained seronegative, and no clinical disease was noted (Kasorndorkbua et al. 2003), further confirming the results from the pregnant monkey study. Therefore, the naturally-occurring swine model is limited for HEV pathogenicity studies, although this model has been useful for various other aspects of HEV research (Feagins et al. 2008; Huang et al. 2005).

Avian HEV infection in chickens leads to Hepatitis-Splenomegaly Syndrome including egg drop, regressive ovaries, and serosanguinous abdominal fluid (Meng et al. 2008). The presence of liver gross abnormalities in the chicken model shares similarities with the course of HEV infection and disease in humans allowing a better characterization of HEV pathogenesis in this naturally-occurring chicken model (Billam et al. 2005). Additionally, the chicken model affords the opportunity to mimic the natural route of HEV infection with oronasal delivery of virus inocula (Billam et al. 2005). While the route of virus inoculation affects the timing of seroconversion (i.v.
develops earlier than oronasal), the patterns of seroconversion, viremia, and development of clinical and pathologic lesions are similar to those seen in HEV infection in humans (Billam et al. 2005). In a comparative pathogenicity study utilizing a strain of avian HEV (HEV-VA strain) obtained from a clinically healthy chicken and the prototype avian HEV strain recovered from a diseased chicken, no significant differences were seen in pathogenicity indicating that other unknown factors may also be involved in the HEV pathogenesis (Billam et al. 2009). In another study, avian HEV-VA was capable of inducing histopathologic liver lesions despite failing to elicit a clinical disease in the chicken model (Kwon et al. 2011). With chickens being a naturally-occurring animal model mimicking certain aspects of the human disease, identification of viral and host factors that determine pathogenicity in chickens would serve as a baseline for identifying these same factors in HEV infection in humans. A major drawback for this model is that chickens are not susceptible to infection by mammalian HEV strains.

Rabbits inoculated with human HEV genotypes 1 and 4 failed to produce clinical disease, elevated liver enzymes, or significant histopathologic lesions as seen in rabbits inoculated with rabbit HEV (Ma et al. 2010). Genotype 4 human HEV was capable of infecting only 2 of 9 rabbits, thus confirming the ability for cross-species infection but at an inefficient level (Ma et al. 2010). Genotype 1 human HEV was incapable of eliciting any markers of HEV infection in rabbits, and thus the usefulness of the rabbit model for HEV pathogenicity study remains to be determined.

Molecular biology and virus replication studies

Experimental infections of rhesus and cynomolgus monkeys have been widely used for infectivity and replication studies through intravenous and intrahepatic inoculations (Aggarwal et al. 2001; Krawczynski et al. 2011; Tsarev et al. 1993a) (Table 3). Hepatic expression of HEV
antigens indicating viral replication in conjunction with the detection of HEV RNA in bile and feces were identified in rhesus macaques prior to appearance of gross lesions within the liver parenchyma (Krawczynski et al. 2011). Chimpanzees and rhesus macaques have been successfully used to determine the infectivity of infectious cDNA clones of HEV via intrahepatic inoculation of RNA transcripts synthesized from cloned cDNA genome of HEV (Emerson et al., 2001). The non-human primate model has been indispensable in studying HEV replication especially during the early days after the virus discovery.

The naturally-occurring swine model has played important roles in understanding the molecular mechanism of HEV replication. By using the swine model, a genotype 3 swine HEV infectious clone was established without the need of an *in vitro* cell culture system (Huang et al. 2005; Krawczynski et al. 2011). Intrahepatic inoculation of pigs with capped RNA transcripts from HEV infectious clones provided a unique means to study the effect of in vitro genetic manipulation of HEV genome on virus replication and pathogenicity (Huang et al. 2005). The identification of an attenuated mutant HEV (pSHEV-1) led to the characterization of specific amino acid residues (F51L, T59A, and S390L) in the capsid protein that are important for virus attenuation in the swine model (Cordoba et al. 2011). Both the T59A and S390L mutations drastically lowered viral RNA loads in intestinal contents, bile, and liver, and shortened the duration of fecal viral shedding (Cordoba et al. 2011).

The hypervariable region (HVR) in ORF1 of HEV varies considerably between different HEV genotypes and among HEV strains. By using the swine model, it was demonstrated that the HVR of HEV is dispensable for HEV infectivity, although a near complete deletion of the HVR attenuated the virus (Pudupakam et al. 2009). By using the chicken model, the impact of complete HVR deletion on virus infectivity was further tested using an avian HEV mutant with a
complete HVR deletion. Although the HVR deletion mutant was still replication competent in LMH chicken cells in vitro, the complete HVR-deletion mutant resulted in a loss of avian HEV infectivity in the chicken model (Pudupakam et al. 2011).

The small ORF3 protein of HEV is multifunctional and involved in virus replication in vivo (Huang et al. 2007; Kenney et al. 2012). Using a homologous naturally-occurring pig model, the authentic initiation site for HEV ORF3 translation was identified as the third in-frame AUG codon in the junction region (Huang et al. 2007). A mutant virus with a mutation in the third in-frame AUG completely abolished the virus infectivity in the pig model, whereas mutations in the first and second in-frame AUG codons in the junction region did not affect the virus infectivity in pigs (Huang et al. 2007). Furthermore, by utilizing the naturally-occurring chicken model, it was demonstrated that the PSAP motif in the ORF3 of avian HEV is involved in particle release from the cell and viral fecal shedding (Kenney et al. 2012). Taken together, in the absence of an efficient cell culture system for HEV, these naturally-occurring swine and chicken models are important for studying the molecular mechanisms of HEV replication.

Cross-species HEV infection studies

Rhesus monkeys are widely used in HEV cross-species infection studies due to the ability to be infected by all four genotypes of human HEV and development of virologic, pathologic, and serologic characteristics consistent with HEV infection (Krawczynski et al. 2011) (Table 3). Two rhesus monkeys and one chimpanzee were successfully infected with a genotype 3 swine HEV resulting in acute viral hepatitis, seroconversion to anti-HEV antibodies, fecal virus shedding, viremia, and slight elevations in ALT, thus serving as experimental surrogates for human HEV infections (Meng et al. 1998). Rhesus macaques were also successfully infected with an Indian
strain of genotype 4 swine HEV as evidenced by viremia and seroconversion to anti-HEV antibodies (Arankalle et al. 2006). In addition, cynomolgus monkeys were readily infected with rabbit HEV with the development of viremia, elevated liver enzymes, presence of fecal virus shedding, and seroconversion to HEV antibodies indicating that, similar to other genotype 3 HEV strains, the rabbit HEV may likely infect humans (Liu et al. 2013).

The swine model has been used to study the cross-species infection and susceptibility of human HEV. Specific-pathogen-free pigs are readily infected by the genotypes 3 and 4 strains of human HEV (Cordoba et al. 2012; Feagins et al. 2008; Meng 2003). In infected pigs, seroconversion occurred by 28 dpi and fecal viral shedding and viremia occurred by 7-56 dpi indicating that swine serve as an excellent model for human HEV infection (Feagins et al. 2008). By using the swine model, it was demonstrated that intergenotypic chimeric HEVs with the genotype 4 human HEV capsid gene cloned in the backbone of genotype 3 swine HEV are infectious in pigs, furthering confirming the zoonotic nature of genotypes 3 and 4 HEV (Feagins et al. 2011). Swine were also shown to be susceptible to infection by rabbit HEV, but resistant to infection with the rat HEV (Cossaboom et al. 2012). The pig model will be important in identifying the genetic elements in the virus genome that determine the cross-species HEV infection between humans and swine.

Under experimental conditions, avian HEV from chickens has been demonstrated to cross species barriers and infect turkeys (Sun et al. 2004); however, attempts to infect rhesus monkeys with avian HEV were unsuccessful (Huang et al. 2004), suggesting that avian HEV has a limited host range and is not zoonotic. Turkeys inoculated with avian HEV seroconverted by 4-6 weeks post-infection, viremia was detected by 2-6 weeks, and a control negative turkey became infected
by direct contact (Sun et al. 2004) indicating that the infection is readily transmissible in a new host.

Rodents including Balb/c nude mice (Huang et al. 2009), C57BL/6 mice (Li et al. 2008), Wistar rats (Li et al. 2013), and Mongolian gerbils (Li et al. 2009) may potentially serve as animal models for some aspects of HEV study; however, few transmission studies in these species resulted in productive infections, and some of these reports have not yet been independently confirmed.

Rabbits serve as a natural host for genotype 3 HEV and are susceptible experimentally to infection by human HEV genotype 4 (Liu et al. 2013). Experimental HEV inoculations in rabbits yield viremia with fecal virus shedding, seroconversion, and mild hepatic lesions consistent with HEV infection (Ma et al. 2010). The rabbit strain of HEV was shown capable of infecting swine and rhesus monkeys indicating the ability of rabbit HEV to infect across species barrier (Cossaboom et al. 2012). The susceptibility to cross-species infection of rabbits by human HEV genotypes 3 and 4 indicated the potential use of rabbits as an alternate model for human HEV.

Future Perspectives

Despite recent advances in the genetic identification of novel animal strains of HEV, characterization of the course of infection and disease in a variety of animal hosts, the underlying molecular mechanisms of HEV replication, pathogenesis and cross-species infection remain largely unknown in part due to the lack of an efficient cell culture and a practical animal model for HEV. Several naturally-occurring animal models such as swine, chicken and rabbit have recently been developed and shown to be useful for various aspects of HEV studies. However, there still lacks a reproducible hepatic disease animal model for the study of human HEV pathogenesis. For examples, the observed severe and fulminant hepatitis E in pregnant women could not be
reproduced in pregnant pigs or pregnant rhesus monkeys. Such an animal model will be critical in identifying the underlying mechanisms of fulminant hepatitis E during pregnancy. Currently, chronic and persistent HEV infection is an emerging and significant clinical problem in immunocompromised individuals such as organ transplant recipients. Unfortunately, a useful animal model that can mimic chronic HEV infection is still lacking, and thus hindering our understanding the mechanism for progression into chronicity and the progress of developing effective antivirals against chronic hepatitis E. Clearly, identifying additional animal models that can more adequately mimic the course of HEV infection and outcomes of disease in humans are important for future HEV research. The expanding host range of HEV offers the opportunities to identify potential new animal strains of HEV that could lead to the development of better naturally-occurring animal model(s) for HEV. Therefore, genetic identification and characterization of additional novel animal strains of HEV are warranted, and development of an efficient cell line to propagate different strains of HEV in vitro will be the key for future development of a cost-effective modified live-attenuated vaccine against HEV.

Acknowledgements

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References


TABLES

Table 1: Animals with Naturally Occurring HEV Infections

<table>
<thead>
<tr>
<th>Species</th>
<th>HEV Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetically confirmed infection</td>
<td></td>
</tr>
<tr>
<td>Domestic Swine</td>
<td>3, 4</td>
</tr>
<tr>
<td>Wild boar</td>
<td>3, 4, unclassified new genotype</td>
</tr>
<tr>
<td>Deer</td>
<td>3</td>
</tr>
<tr>
<td>Chicken</td>
<td>Avian HEV genotypes 1, 2, 3</td>
</tr>
<tr>
<td>Rabbit</td>
<td>3</td>
</tr>
<tr>
<td>Rat</td>
<td>unclassified new genotype, 3 (not independently confirmed)</td>
</tr>
<tr>
<td>Mongoose</td>
<td>3</td>
</tr>
<tr>
<td>Moose</td>
<td>unclassified new hepevirus</td>
</tr>
<tr>
<td>Ferret</td>
<td>unclassified new hepevirus</td>
</tr>
<tr>
<td>Bat</td>
<td>unclassified new hepevirus</td>
</tr>
<tr>
<td>Cutthroat Trout</td>
<td>unclassified new hepevirus</td>
</tr>
<tr>
<td>Serological evidence of infection</td>
<td></td>
</tr>
<tr>
<td>Rhesus Macaque</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cynomolgus</td>
<td>Unknown</td>
</tr>
<tr>
<td>Goat</td>
<td>Unknown</td>
</tr>
<tr>
<td>Sheep</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cattle</td>
<td>Unknown</td>
</tr>
<tr>
<td>Species</td>
<td>HEV genotypes</td>
</tr>
<tr>
<td>------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td><strong>Non-Human Primates:</strong></td>
<td></td>
</tr>
<tr>
<td>Cynomolgus monkey</td>
<td>Human 1, 2</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>Human 1, 2, 3, 4</td>
</tr>
<tr>
<td></td>
<td>Swine 3, 4</td>
</tr>
<tr>
<td></td>
<td>Avian HEV</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>Human 1, 2, 3, 4</td>
</tr>
<tr>
<td></td>
<td>Swine 3, 4</td>
</tr>
<tr>
<td>Tamarins</td>
<td>Human 1, 2</td>
</tr>
<tr>
<td>Owl Monkey</td>
<td>Human 1, 2</td>
</tr>
<tr>
<td>Vervet</td>
<td>Human 1, 2</td>
</tr>
<tr>
<td>Squirrel Monkey</td>
<td>Human 1, 2</td>
</tr>
<tr>
<td>Patas</td>
<td>Human 1, 2</td>
</tr>
<tr>
<td>Domestic Swine</td>
<td>Human 3, 4</td>
</tr>
<tr>
<td></td>
<td>Human 1</td>
</tr>
<tr>
<td></td>
<td>Human 2</td>
</tr>
<tr>
<td></td>
<td>Rabbit HEV</td>
</tr>
<tr>
<td></td>
<td>Avian HEV</td>
</tr>
<tr>
<td></td>
<td>Rat HEV</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Human 4</td>
</tr>
<tr>
<td></td>
<td>Human 1</td>
</tr>
<tr>
<td></td>
<td>Swine 4</td>
</tr>
<tr>
<td>Chicken</td>
<td>Human 1</td>
</tr>
<tr>
<td></td>
<td>Swine 3</td>
</tr>
<tr>
<td></td>
<td>Avian HEV</td>
</tr>
<tr>
<td>Turkey</td>
<td>Avian HEV</td>
</tr>
<tr>
<td>Lamb</td>
<td>Human 1</td>
</tr>
<tr>
<td><strong>Rodents:</strong></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Human 1, 2, 3, 4</td>
</tr>
<tr>
<td></td>
<td>Swine 3</td>
</tr>
<tr>
<td></td>
<td>Avian HEV</td>
</tr>
<tr>
<td>Balb/C mice</td>
<td>Swine 4</td>
</tr>
<tr>
<td>C57BL/6 mice</td>
<td>Human 1</td>
</tr>
<tr>
<td></td>
<td>Swine 3,4</td>
</tr>
<tr>
<td>Mongolian Gerbil</td>
<td>Swine 4</td>
</tr>
</tbody>
</table>
**Table 3: Applications of available animal models including naturally occurring animal models for various aspects of HEV studies**

<table>
<thead>
<tr>
<th>Species</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chimpanzee</td>
<td>Pathogenesis, Molecular Biology and Virus Replication, Cross-species Infection</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>Vaccine, Pathogenesis, Molecular Biology and Virus Replication, Cross-species Infection</td>
</tr>
<tr>
<td>Cynomolgus Monkey</td>
<td>Vaccine, Pathogenesis, Molecular Biology and Virus Replication, Cross-species Infection</td>
</tr>
<tr>
<td>Owl Monkey</td>
<td>Cross-species Infection</td>
</tr>
<tr>
<td>Rodents</td>
<td>Vaccine, Cross-species Infection</td>
</tr>
<tr>
<td>Swine</td>
<td>Vaccine, Pathogenesis, Molecular Biology and Virus Replication, Cross-species Infection</td>
</tr>
<tr>
<td>Chicken</td>
<td>Vaccine, Pathogenesis, Molecular Biology and Virus Replication, Cross-species Infection</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Vaccine, Pathogenesis, Cross species Infection</td>
</tr>
</tbody>
</table>
Figure 1: A phylogenetic tree based on the full-length genomic sequences with genotype classification of known animal strains of HEV. Sequence alignment was completed using ClustalW, MEGA version 5.2 (www.megasoftware.net) for the phylogenetic tree, and the tree was constructed using the neighbor-joining method with the maximum composite likelihood method for evolutionary distances corresponding to each branch length and the units of the number of base substitutions per site. Each of the four known genotypes (1-4) is labeled, with representative strains included for each species, and novel strains from ferret, bat, rat, avian, and fish identified individually as separate new putative genotypes or species.
Chapter III: Hepatitis Virus Infections in Poultry

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Summary

Viral hepatitis in poultry is a complex disease syndrome caused by several viruses belonging to different families including avian hepatitis E virus (HEV), duck hepatitis B virus (DBHV), duck hepatitis A virus (DHAV-1, -2, -3), duck hepatitis virus types 2 and 3, fowl adenoviruses (FAdV), and Turkey hepatitis virus (THV). While these hepatitis viruses share the same target organ, the liver, they each possess unique clinical and biological features. In this article, we aim to review the common and unique features of major poultry hepatitis viruses in an effort to identify the knowledge gaps and aid the prevention and control of poultry viral hepatitis. Avian HEV is an Orthohepevirus B in the family Hepeviridae that naturally infects chickens and consists of 3 distinct genotypes worldwide. Avian HEV is associated with Hepatitis-Splenomegaly syndrome or Big Liver and Spleen Disease in chickens, although the majority of the infected birds are subclinical. Avihepadnaviruses in the family of Hepadnaviridae have been isolated from ducks, snow geese, white storks, grey herons, cranes, and parrots. DHBV evolved with the host as a noncytopathic form without clinical signs and rarely progressed to chronicity. The outcome for DHBV infection varies by the host’s ability to elicit an immune response and is dose and age dependent in ducks, thus mimicking the pathogenesis of human HBV infections and providing an excellent animal model for human HBV. DHAV is a picornavirus that causes a highly contagious virus infection in ducks with up to 100% flock mortality in ducklings under 6 weeks of age, while older birds remain unaffected. The high morbidity and mortality have an economic impact on intensive duck production farming. DHV types 2 and 3 are astroviruses in the family of Astroviridae with similarity phylogenetically to turkey astroviruses implicating the potential for cross-species infections between strains. DAstV causes acute, fatal infections in ducklings with a rapid decline within 1-2 hours and clinical and pathological signs virtually indistinguishable from
DHAV. DAstV-1 has only been recognized in the United Kingdom and recently in China, while DAstV-2 has been reported in ducks in the United States. Fowl adenoviruses (FAdV), the causative agent of Inclusion Body Hepatitis (IBH), is a group I avian adenovirus in the genus *Aviadenovirus*. The affected birds have a swollen, friable and discolored liver, sometimes with necrotic or hemorrhagic foci. Histological lesions include multifocal necrosis of hepatocytes and acute hepatitis with intranuclear inclusion bodies in the nuclei of the hepatocytes. Turkey Hepatitis Virus (THV) is a picornavirus that is likely the causative agent of Turkey viral hepatitis (TVH). Currently there are more questions than answers about THV, and the pathogenesis and clinical impacts remain largely unknown. Future research in viral hepatic diseases of poultry is warranted to develop specific diagnostic assays, identify suitable cell culture systems for virus propagation, and develop effective vaccines.

**Key Words/Index Terms**

Avian hepatitis E (avian HEV); Duck hepatitis B virus (DHBV); Duck hepatitis virus type 2 (DAstV-1, DAstV-2); Duck hepatitis A (DHAV); Fowl Adenovirus (FAdV); Turkey hepatitis virus (THV)
Introduction

Viral hepatitis in poultry is an important disease syndrome caused by a number of viruses including avian hepatitis E virus (avian HEV), duck hepatitis B virus (DBHV), duck hepatitis A virus (DHAV), duck hepatitis virus types 2 and type 3, fowl adenoviruses, and Turkey hepatitis virus. While these avian hepatitis viruses have the same target organ, the liver, they are very different in virus biology and pathogenesis as each of these viruses belongs to a different family. Therefore, understanding the common and unique features of these hepatitis viruses in poultry will aid the diagnosis and differential diagnosis of viral hepatitis in poultry and help develop more effective flock management, preventive and control measures.

Avian Hepatitis E Virus (Avian HEV)

History: A disease, referred to as Big Liver and Spleen (BLS) Disease (1, 2), was first reported in chickens in Australia (3). The estimated impact of BLS on Australia chicken producers was a loss of 8 eggs per hen in affected broiler-breeder flocks leading to a total 2.8 million Australian dollar loss to the industry and nearly 50% of all flocks affected. A similar disease, referred to as Hepatitis-Splenomegaly (HS) syndrome, was also reported in layer and broiler-breeder chickens in the United States and Canada in 1991(1, 2). The disease presented as a decrease in overall mortality and egg production, while field veterinarians reported serosanguinous abdominal fluid or clotted blood in the abdomen and enlarged livers and spleens at necropsy (3). Initially, attempts to link the syndrome to any specific agent were unsuccessful (3-5) and only one outbreak was able to be linked to a strain of Campylobacter spp (3). Avian hepatitis E virus (avian HEV) was first identified from chickens with HS syndrome in the United States in 2001 (1). HS syndrome and BLS are caused by variants of the same avian HEV (1, 2, 6, 7).
**Classification:** The BLS virus from Australia shares approximately 80% of nucleotide sequence identity with avian HEV from HS syndrome (2, 6), while all known strains of avian HEV worldwide share approximately 60% of nucleotide sequence identity and are divided into three separate genotypes (genotypes 1-3) (6). A recent ICTV proposal includes division of the HEV strains among three genera: *Orthohepeivirus A* which includes all genotypes 1-4 strains that infect humans, camels, wild boars, deer, rabbits, rats, and mongooses, *Orthohepeivirus B* including all avian HEV strains, *Orthohepeivirus C* consisting of all ferret and most rat HEV strains, *Orthohepeivirus D* including variants of bat HEV strains, and *Piscihepeivirus* including all strains of cutthroat trout hepeviruses (8).

**Seroprevalence and transmission:** Chickens are the only known reservoir for avian HEV infection under natural conditions. Approximately 71% of chicken flocks within the United States are seropositive to avian HEV based on a survey of 1,276 chickens from 76 flocks within five states (7). The virus appears to spread easily within and between flocks via the fecal-orale route of transmission (3). In an age-dependent manner, 17% of young chickens (under 18 weeks of age) are positive for anti-HEV antibodies, while 36% of adult chickens are seropositive (7, 9). Avian HEV infections in chickens have been described throughout the United States (1, 9, 10), Canada (7, 11), the United Kingdom (12), Australia (13), Taiwan, China (14), and Russia (15). Caged leghorn chickens are frequently affected with reoccurrence on many farms due to circulation of the virus within the flock (5, 15). Broiler breeder hens, dual-purpose hens, and smaller flocks may be infected by avian HEV with sporadic mortality especially when housed on litter allowing for the accumulation and ease of transmission between birds (7). In a prospective transmission study monitoring 14 seronegative chickens beginning at 12 weeks of age, all birds seroconverted to anti-HEV antibodies by 21 weeks of age (9). Large amounts of virus are shed in feces of chickens.
infected experimentally by avian HEV; therefore, feces likely serve as the main source for virus spread within the flock (3, 16). Other routes of transmission including aerosol, vertical, vector-borne, or mechanical carrier have not been demonstrated in natural or experimental avian models (16).

**Clinical signs and pathobiology:** The mortality rate for chickens infected with avian HEV genotypes 1-3 ranges from 0.3% to 1.0% of the overall flock (3); therefore, many birds carry subclinical avian HEV infections, thus expediting and masking transmission between birds. Few birds show clinical signs prior to acute death (3-5, 7, 9); however, egg drop with up to 20% of hens presenting with reduced production and hepato-splenomegaly are described in some outbreaks (5), while others do not appear to affect overall production. The incubation period ranges between 1-3 weeks in birds infected via oronasal route in an experimental setting (17). In the field, broiler breeder and laying hens experience the highest incidence of mortality at ages 30-72 weeks, with mortality typically lasting several weeks during the mid-production period for these flocks (7, 16, 18, 19). Clinical signs in BLSV-infected flocks in Australia have more severe infections with birds presenting with pale wattles and combs, pasty droppings, soiled vents, anorexia, depression, and elevations in egg drop and mortality for 3-4 weeks (3, 5, 18-20). While the eggs produced in infected chickens retain commercial value based on comparable quality, fertility, and hatchability, the shells themselves are lacking pigment and thin (3).

The appearance of IgG antibodies following avian HEV infection characterizes the humoral immune response in chickens (17, 21), while knowledge is lacking as to the cell-mediated immune response. Following inoculation via intravenous or oronasal route, chickens seroconvert between 1-4 weeks post-infection (21). The capsid protein (ORF2) of avian HEV shares conserved antigenic epitopes with swine and human HEV capsids and elicits a protective immune response
against avian HEV infection (22). In vitro analysis of recombinant HEV capsid proteins demonstrates cross-reactivity between avian HEV capsid and antisera raised against human HEV (Sar-55 genotype 3 strain), swine HEV (Meng strain), and human HEV (US-2 genotype 2 strain) (22-24).

Clinico-pathological parameters of liver disease including elevations in aspartate aminotransferase (AST), albumin/globulin (A/G) ratios, or bile acids are not present in chickens experimentally infected with avian HEV; however, lactate dehydrogenase (LDH) levels varied with progression of virus infection with intravenously-infected chickens exhibiting peak LDH levels one week post-infection (wpi), and oronasally-infected chickens exhibiting peak LDH elevations from 1-4 and 6 wpi prior to returning to baseline level by 7 wpi (17). At necropsy, birds infected with avian HEV display regressed ovaries, enlarged, hemorrhagic, friable, and necrotic livers with subcapsular or capsular hematomas (Fig. 1), enlarged spleens, and serosanguinous fluid within the abdominal cavity (1, 3, 7, 21). Experimental avian HEV infections in chickens display similar gross lesions at necropsy with nearly 25% presenting with enlarged hemorrhagic livers (17). Microscopic evaluations of natural and experimental infections in chickens consistently show inflammatory cellular infiltrations within the parenchyma of the liver and a lymphocytic periphlebitis and phlebitis in the liver (17). Lesions vary from multifocal to extensive areas of necrosis, hemorrhage, heterophillic and mononuclear inflammatory infiltrates as well as segmental lymphocytic and plasma cell infiltrates around the portal veins. Commonly, hepatocytes are separated by interstitial accumulation of eosinophillic amyloid deposits (4, 17).

The pathogenesis of avian HEV in chickens remains mostly unknown. Presumably, the virus enters the host by direct contact with contaminated feces via oronasal inoculation and travels to the gastrointestinal tissues where virus replication takes place as the primary site (17, 25).
detection of avian HEV in experimentally infected chickens in the colon, cecum as early as 5 days post-infection (dpi), jejunum by 20 dpi, ileum by 7 dpi, duodenum by 20 dpi, and cecal tonsils by 35 dpi further confirms a gastrointestinal site of primary virus replication. Following entry and replication in the GI tissues, the virus travels to the liver as a secondary site of virus replication based on experimental infections of mammalian HEV in primates and swine, and is released from hepatocytes into the gallbladder (26). The gallbladder expels contents containing infectious avian HEV particles during normal digestion into the small intestines where the virus is capable of traveling throughout to finally be expelled in excrement into the environment. The exact mechanisms of avian HEV replication and release remain largely unknown however, and studies thus far rely solely upon the ability to detect infectious virus as confirmation of virus replication (16, 27). Understanding the importance of these extrahepatic sites of virus replication and their roles in transmission are crucial in understanding and diminishing the spread of virus.

**Cross-species infection:** Avian HEV is limited to chickens under field conditions; however, turkeys were successfully infected with avian HEV under experimental conditions as evidenced by seroconversion to anti-HEV antibodies, fecal virus shedding, and viremia (21). The inoculated turkeys seroconverted at 4-6 wpi with viremia detectable by 2-6 weeks. In addition, contact between control and infected turkeys allowed for transmission of the virus indicating the ease of transmission in birds (21). Subsequent attempts to infect mice (3) and rhesus macaques with avian HEV (28) were unsuccessful. Experimentally, all ages of chickens are susceptible to infection with avian HEV by intravenous and oronasal routes of inoculation (17, 21, 29). Embryonic chicken eggs; however, were only susceptible to avian HEV infection when inoculated intravenously (30).
**Prevention and control:** A vaccine against avian HEV is not yet available. Avian HEV capsid protein has been used to induce protective immunity when challenged with avian HEV in chickens (23). Chickens immunized with keyhole limpet hemocyanin-conjugated avian HEV capsid (KLH) peptides did not induce protection when challenged with avian HEV (31). However, chickens immunized with recombinant avian HEV capsid antigens had complete protection against avian HEV challenge. Identifying specific B-cell epitopes within avian HEV capsid protein is useful in future vaccine design and development of diagnostic immunoassays (24). In the lack of a vaccine, strict biosecurity and better hygiene practices on the farm help prevent the spread of avian HEV infection in flocks.

**Unanswered questions and future prospective:** Even though avian HEV was discovered more than a decade ago, there remain many unanswered questions. Due to the lack of a cell culture system to propagate avian HEV, the biology and pathogenesis of the virus are still poorly understood. The lack of a cell culture system significantly hinders the development of an effective and practical vaccine against avian HEV. The extent of infection and genetic variations of avian HEV from chickens worldwide are also unknown, as thus far only three genotypes were identified from chickens in just a few countries. Although most avian HEV infections are subclinical, the effect of co-infection between avian HEV and other poultry pathogens on disease manifestation is unknown. A commercial diagnostic assay for avian HEV is still lacking, even though homemade RT-PCR and ELISA assays have been used for research purpose. Future research is warranted to identify suitable cell culture systems that can efficiently propagate avian HEV, to develop avian HEV-specific diagnostic assays, to understand the effect of avian HEV co-infection with other agents in chicken flocks, and to develop an effective vaccine against avian HEV.
Duck Hepatitis B Virus (DHBV)

**History:** Following the discovery of human hepatitis B virus (HBV) in 1970 (32) and subsequent discovery of HBV-like viruses in numerous primate species (33-36) and non-primate species (37-39), the first Avihepadnavirus was identified in Pekin ducks (*Anas platyrhynchos forma domestica*) in 1980 (40, 41). Avihepadnaviruses have now been isolated from snow geese (*Anser caerulescens*) (42), white storks (*Ciconia ciconia*) (43), grey herons (*Ardea cinerea*) (44), cranes (*Grus genus*) (45), and parrots (*Psittacula krameri*) (46). The virus is detectable in many commercially bred flocks. The narrow host range of Avihepadnaviruses limits virus infection to each respective species with little cross-species infectivity including in chickens or other Anseriformes such as Muscovy ducks (*Cairina moschata*) (47). Except in cases in which aflatoxin carcinogens are present, Duck hepatitis B virus (DHBV) does not induce clinical signs of pathogenicity and only rarely progress into chronicity and liver cancer (48); therefore, the virus is assumed to have evolved with the host as a noncytopathic form (49).

**Classification:** *Hepadnaviridae* family consists of *Orthohepadnavirus* and *Avihepadnavirus* genera for those viruses infecting mammals and birds, respectively (50). *Avihepadnaviruses* include duck hepatitis B (DHBV) (41), heron hepatitis B (HHBV) (44), Ross goose hepatitis (RGHV) (42), stork hepatitis B (STHBV) (43), and parrot hepatitis B (PHBV) (46) viruses. DHBV is the most characterized virus of the genera and shares approximately 40% nucleotide sequence identity with human HBV (50). *Orthohepadnaviruses* include human HBV, non-human primate hepatitis viruses such as chimpanzee (ChHBV) (34), gorilla (GoHBV) (51), orangutan (OuHBV) (52), woolly monkey (WMHBV) (33, 36), and gibbon (GiHBV) (53), rodent hepatitis viruses such as ground squirrel (GSHV) (39), arctic squirrel (ASHV) (38), and woodchuck (WHV) (37), as well as bat hepadnavirus from Burmese bats (BtHV) (54). All 10
genotypes (A-J) of human HBV varied with more than 8% nucleotide sequence diversity (50, 55). The non-human primate strains carry 7-9% divergence (50) and form geographical clusters (56), while sequence divergence with human HBV strains ranges from 10-15% for most strains aside from WMHBV, which carries 28% nucleotide sequence diversity with other primate strains (36). Non-primate strains of HBV constitute a separate species of Orthohepadnavirus with sequence identities varying by 53% for BtHV, 37% for ASHV, 45% for GSHV, and 30% for WHV (50) with human HBV.

Clinical signs and pathobiology: The pathogenesis of DHBV in ducks mimics human HBV infections with an ability to lead to an acute and transient infection or a chronic infection. Young ducks develop an age-dependent and dose-dependent predisposition to carrying a persistent infection, while older ducks tend to clear the virus infection transiently (57). Natural infection of DHBV occurs by vertical transmission from an infected hen to the embryonic eggs through the bloodstream and subsequent infection of embryonic hepatocytes at day 6 of development resulting in infected offspring (58). Viral replication occurs in the yolk sac and developing embryonic hepatocytes resulting in a congenital infection (58, 59) followed by continued virus replication in the hepatocytes and a small percentage in pancreatic, kidney, and splenic cells (60) of infected ducks and geese with a very high serum viral titer \((10^{10} \text{ virions or } 10^{13} \text{ viral particles per ml})\) when persistent infection is established (49, 61). Additionally, it has been shown that only one genome copy of the virus is needed to successfully infect a duckling and spread to nearly full hepatocyte infection within 14 days post-inoculation (62). Chinese domestic ducks, American Pekin ducks, and a few geese species are infected with DHBV with poor efficiency outside the narrow host range (63). Hepadnaviruses are cell type- and host-specific allowing for infections in only a few closely related species. Infected adult ducks face a far different outcome by carrying a transient
acute infection that is cleared by neutralizing antibodies. These outcomes mimic the range of human HBV infections with neonates resulting in persistent infections, adults resulting in transient infections, and those persistently infected resulting in varying degrees of severity from mild chronic hepatitis to a chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma (60).

The outcome for DHBV infection varies based on the host’s ability to elicit an immune response. Variability in non-cytopathic viruses include the dose of inoculum, viral replication, and cell, tissue, and host tropism being balanced with the humoral and cell-mediated immunity of the host (60, 64). It has been suggested that the number of lymphoid cells present or the overall maturity of the immune system affects the ability to elicit a virus-clearing immune response resulting in the disparity seen between neonates and adults infected with DHBV (60). The age of the duck with neonates primarily being persistently infected with DHBV and the dose of inoculum resulting in a more severe infection consistently supports this theory (57, 59). In young ducks inoculated experimentally with DHBV, persistent infection is evidenced by a mild mononuclear cellular infiltration within the portal tracts of the liver, but no lobular hepatitis (57). In one study, goose and duck embryos exhibited mononuclear and heterophillic cell infiltrations perivascularly without severe inflammation (65). GHBV-infected goose embryos predominantly exhibited hepatic bile ductular cell proliferation and varying degrees of hepatocellular vacuolization. Adult ducks experimentally infected with DHBV clear the infection without pathological changes in most cases. However, in one study, one out of five adult ducks developed severe chronic hepatitis and a prolonged infection (57). DHBV results in little clinical disease or lesions for congenitally infected or acute experimentally infected birds (65), unlike its mammalian counterparts. Hepatocellular carcinoma as an outcome has not been established with congenital or experimental
DHBV infections in ducks, likely due to the short lifespan and period of 25-30 years required for adequate chronic inflammation, cell regeneration, cirrhosis, and finally HCC in humans (60).

In ducks infected with DHBV, anti-DHBV surface antibodies are detectable by 17 days post-inoculation (dpi). By 40 dpi, serum containing DHBV antibodies was capable of neutralizing DHBV infections in 1-day-old ducklings (66, 67). In addition, ducks ranging from 3-8 weeks of age were seropositive for anti-DHBV antibodies in 20-40% of cases (68). Anti-DBHV core antibodies are present in the sera of persistently infected ducklings by 7-10 dpi; however, there is no clearance of virus infection, and anti-DBHV surface antibodies fail to develop (57). Increasing dosages of inoculum in 4 month-old ducks both increased the levels of detectable antibodies with a correlative level of infection of the liver as well as shortening the time to serum antibody appearance (57). DHBV and WHV infections lead to >95% of hepatocytes participating in viral replication, and clearance occurred coincidentally with anti-viral surface antibody increases in the serum (57, 69). In both cases, only minimal cellular infiltration and liver enzyme elevations were observed. IgM antibodies predominate early in the immune response with a switch to two distinct forms of IgY later in the response. Likewise, a cell-mediated immune response specifically by cytotoxic T-cells is necessary for complete viral clearance during persistent DHBV infection (60). In studies completed in transgenic mice, HBV-specific CTLs lead to apoptosis of hepatocytes leading to clearance of cells expressing HBV antigens (70). Non-cytolytic mechanisms of viral clearance likely play a role with HBV infections as well. In HBV transgenic mice, IFN-γ and TNF-α secretions were capable of suppressing HBV surface antigens in hepatocytes (71). Previously, HBV mRNA expression was hampered by IFN-γ and TNF-α in transgenic mice (70, 71).

**Cross-species infection:** Hepadnaviruses are generally considered as host specific; however, it is well established that recombination occurs between genotypes of human HBV (72)
as well as between human and ape and different non-human primate variants (73). Much like mammalian hepadnaviruses, the *avihepadnaviruses* also infect a narrow host range. DHBV is not able to infect the Muscovy duck or chickens and remains limited in susceptibility to only a few species of ducks or geese (47). CHBV (crane) is capable of infecting primary duck hepatocytes (45), although the full clinical spectrum of infection in ducks *in vivo* and the ability to establish chronicity are uncertain (63). HHBV (grey heron) only inefficiently infects primary duck hepatocytes (PDHs) *in vitro* and does not infect ducks *in vivo* (44). Alterations in the HHBV-specific preS domain of the large envelope protein (L protein) with the appropriate sequence from DHBV allows for efficient infection of PDHs by HHBV due to conservation of the biological function of this region (63, 74). The same was shown to be true for woolly monkey hepatitis B virus for use in infecting human hepatocytes (63, 75). Evidence is lacking for zoonotic transmission of *avihepadnaviruses* to humans.

*Experimental animal models for human HBV*: DHBV as a model system for the study of human HBV is well established and particularly advantageous due to the ability to reproduce infection in ducks and the use of PDHs for both *in vitro* and *in vivo* molecular studies. HBV and DHBV share many characteristics including being partially double-stranded DNA viruses that use an RNA intermediate with reverse transcription as a replication strategy (76, 77), overlapping open reading frames (ORFs) in viral DNA genomes (63), infections primarily occurring in hepatocytes, and extensive similarities in genomic organization, biological characteristics, and virus replication life cycles. The clinical syndrome between ducks and humans infected with HBV varies considerably however, with ducks exhibiting no clinical signs and remaining healthy despite establishing a chronic persistent virus infection and transmission to subsequent progeny, while in humans liver injury and either hepatocellular carcinoma or cirrhosis of the liver ensues due to
chronic HBV infection (63). Adult birds infected with DHBV also clear the virus with no outward signs of infection or long-lasting effects, while humans develop acute or fulminant hepatitis without elimination. Due to the ability to transfect cloned DHBV into a chicken hepatoma cell line (LMH) (78), mutations to the genome regarding replication strategies, host tropism, treatments, and vaccination strategies can now be studied (63) using the duck model for human HBV infections.

**Unanswered questions and future prospective:** Despite providing a valuable model for the study of human HBV infection, DBHV infections in ducks are unable to reproduce all aspects of infection and pathogenesis including the typical clinical syndrome, immunopathology, and progression to chronic infection and hepatocellular carcinoma. The mechanisms underlying chronic infections are poorly understood and necessary in order to devise future antiviral therapies to ameliorate infections. In addition, the natural course of virus infection characterized by cyclical clearance and reinfection paired with individualistic immune responses to infection complicates our understanding of the pathogenesis and leads to vast differences in therapeutic responses. The effect of a co-infection between DHBV and other pathogens is not understood and long-term treatment strategies cannot be replicated in the duck model for use in human HBV patients. Thus far, very little research has focused on HBV within each avian species nor the interaction and opportunity for cross-species infection. The variable clinical pathology and disease syndrome of HBV in domestic and wild birds warrants further investigation. Lastly, the potential for cross-species infection by hepadnaviruses between humans and mammalian and avian species highlights the need for additional research and surveillance in this poorly understood arena.
**Duck Hepatitis A Virus (DHAV-1, DHAV-2, and DHAV-3)**

**DHAV classification and characteristics:** DHAV is a member of the *Picornaviridae* family in a new genus *Avihepatovirus* as classified by ICTV and is referred to as “duck virus hepatitis A” informally. This new genus contains all three serotypes of duck hepatitis A virus, namely DHAV-1, DHAV-2 from Taiwan, and DHAV-3 from South Korea and China (79-82). Recently, novel picornaviruses have also been identified in turkeys (avisivirus A, AsV-A) (83) and ducks (84) that are genetically and antigenically related to DHAV. DHAV is a typical picornavirus as a single-stranded, non-enveloped, positive-sense, and polyadenylated RNA virus with a genome consisting of one single ORF encoding a polyprotein flanked by 5’ and 3’ untranslated regions (UTRs).

**Clinical signs and pathobiology:** DHAV-1 is a highly contagious virus in ducks associated with diseases in mallard, Pekin, and Muscovy species (85). In natural infection, young ducklings of 6 weeks of age and younger present with lethargy, ataxia, opisthotonos, and a very rapid death, often taking as little as 1-2 hours (86). Due to the high morbidity within a flock of up to 100%, the vast majority of ducklings will be affected with flock mortality of 95-100% occurring within 3-4 days but older birds are not affected (87). At necropsy, the liver is typically enlarged with petechial and ecchymotic hemorrhages throughout (Fig. 2). Microscopic lesions in the liver include extensive hepatocyte necrosis (Fig. 3), bile duct hyperplasia, hemorrhages, and inflammatory cell infiltrations dispersed throughout (88). The spleen and kidneys often swell with congestion of the blood vessels often apparent. The clinical and pathological lesions are characteristic for DHAV-1 infections and with little information on DHAV-2 and DHAV-3 infections, the clinical presentation for DHAV-2 and -3 is assumed similar (86). One study showed that DHAV-3 induced apoptosis and necrosis of multiple organs including the liver, but the mechanism is not understood.
(88). While no public health significance is noted due to DHAV infections, economically, the highly lethal and rapidly spreading infection may affect intensive duck production farming regions of the world (85). DHAV is controlled by vaccination of breeder ducks with a live-attenuated virus in order to transfer maternal immunity to the hatched ducklings (86) with protection waning over the first 2 weeks of life, but re-immunization with a live-attenuated vaccine at 7-10 days of age allows for protection through the susceptible period (89). Hatchling ducks may also be immunized with a live-attenuated vaccine that confers immunity within 48-72 hours lasting through the susceptible period (90) or passive introduction of antibodies for short-lived immunity (86).

**DHAV diagnosis:** Confirmation of DHAV infection is completed by recovery of virus from the liver of affected ducklings and inoculation of 1-7 day-old naïve ducklings, 10-14 day-old embryonated duck eggs, or primary cultures of duck embryo liver cells (DEL) (86). Ducklings follow a characteristic course of infection with death within 18-48 hours, duck embryos die within 24-72 hours, and DEL cells develop a cytopathic effect characterized by rounding and necrosis of the infected cells (86). Necropsy lesions in embryos follow the natural infection characteristics with stunting and hemorrhaging of the entire body, subcutaneous edema of the abdomen and hind limbs, and focal necrosis of the liver parenchyma. Neutralization assays (91-93) have been described for diagnosing DHAV-1 infections; however they are not typically used as confirmation, and diversity between DHAV isolates limits reactivity with hyperimmune sera (93). Nucleic acid recognition techniques including reverse transcriptase PCR (80, 82, 94-96) and real time RT-PCR (97) have been described for the identification of the 3D gene of DHAV-1 and to identify genotypic variation among isolates.
**Unanswered questions and future prospective:** Despite the lack of public health significance of DHAV-1, -2, and -3 infections, the economic impact on duck production warrants further investigation into the mechanisms of pathogenesis and immunopathology implicated in the infectious disease process. Specifically, the age-dependent differences in clinical outcome and mortality between ducklings and adult ducks is of importance for management and control of outbreaks within the flock. Future research into the differences between genotypes of DHV type 1 and their interaction with one another within the host species will greatly enhance the ability to properly diagnose and prevent outbreaks leading to less mortality within the flock. The extent of infection and genetic variation worldwide is currently unknown, in part due to the lack of a DHAV-specific assay outside of research settings, which limits our full understanding of the economic impact as well as the impact of co-infections on the production industry.

**Duck Hepatitis Virus (DHV II & III)**

**DHV type 2 (DAstV-1, DAstV-2) classification and characteristics:** DHV type II is an Astrovirus as classified by ICTV and is referred to as duck Astrovirus type 1 (DAstV-1) (98). The electron microscopy morphology of DAstV-1 resembles that of a typical Astrovirus with 28-30 nm in diameter (99). Astroviruses are single-stranded, positive-sense, non-enveloped, RNA viruses with three ORFs (1a, 1b, 2) and are capable of infecting numerous mammalian and avian hosts (100). DAstV-1 has been identified from ducks in the United Kingdom (101, 102) and recently in eastern China (103). DHV type III (DAstV-2) is considered as genetically distinct (98, 104), while still a member of Astroviridae family. DAstV-2 has only been reported from ducks in the United States (105). Recently, DAstV-3 (106) and DAstV-4 (107), two distinct astroviruses have been identified in Pekin ducks, Shaoxing ducks and Landes geese and Pekin ducks
respectively. In another study reporting a novel isolate of DAstV in China, a phylogenetic analysis revealed a stronger relationship with turkey astrovirus (TAsTV) type 2, TAsTV-3 and TAsTV/MN/01), thus indicating the potential for cross species infection between astrovirus strains (108). More work is warranted to fully understand these new isolates of astroviruses and their potential for cross species transmission.

**Clinical signs and pathobiology:** DAstV-1 causes an acute, fatal infection in ducklings typically between the ages of 10 days to 6 weeks of age (99). Similarly, DAstV-2 causes an acute infection with losses up to 20% of those immune to DHV type 1 (86, 105). The natural clinical infection is similar to DHAV in that lethargy, ataxia, opisthotonos are commonly seen with a rapid decline to death within 1-2 hours (86, 101). Sick ducklings may present with polydipsia prior to death as the only differentiating sign. At necropsy, ducks present with splenomegaly, swollen pale kidneys with congested vessels, empty, mucous filled alimentary tracts with areas of hemorrhage, petechial hemorrhages in the heart, and petechial to diffusely confluent hemorrhages throughout the liver (99, 109). Microscopic changes are similar to those of DHAV with extensive hepatocyte necrosis, bile duct hyperplasia, hemorrhages, and inflammatory cell infiltrations dispersed throughout, but with a relative increase in bile duct hyperplasia noted (109). Under experimental field conditions (110, 111), DAstV-1 may be protected with a live-attenuated vaccine; however, DAstV-2 infections are routinely prevented with immunity transferred to hatching ducklings using a live-attenuated vaccine (86).

**DAstV-1 and -2 diagnosis:** Diagnosis of DAstV-1 infection is achieved by recovery of the virus from the livers of affected ducklings and inoculation into susceptible ducklings or embryonated chicken or duck eggs resulting in only a 20% mortality of ducks (101) and stunting of embryos with green necrotic livers after 6-10 days of infection (109), which is in stark contrast
with DHAV infections in which the mortality rate is much higher and the progression of disease
much faster. Neutralization assays for the identification of virus as well as cross protection tests
(101) in ducklings in order to distinguish between DAsTV types 1 and 2 have been described, but
neither are used routinely. Serological responses to infection are poor in ducklings and duck
embryos, thus rendering an immunological test not applicable. RT-PCR has been described for
confirmation that DAsTV-1 is indeed an astrovirus (104, 112); however, further analyses have not
been completed. Diagnosis of DAsTV-2 infections are achieved by the inoculation into susceptible
ducklings or the chorioallantoic membrane of 10-day-old embryonated duck eggs resulting in a
20% mortality following 60% morbidity in ducklings and embryonic mortality in 7-10 days (86).
Much like DAsTV-1 infections, embryos exhibit stunting, edema, and skin hemorrhages, but
infection may also result in enlargement of the kidneys, liver, and spleen (86). In cell culture,
DAsTV-1 is reportedly capable of infecting primary chicken embryo liver cell lines with plaques
formed at 5 dpi (113), while DAsTV-2 is capable of detection by immunofluorescence in infected
DEL and duck embryo kidney (DEK) cultures (105). RT-PCR has also been used to confirm the
identification of DAsTV-2 as a distinct astrovirus from DAsTV-1 (104, 112).

**Unanswered questions and future prospective:** The mechanisms leading to pathogenesis
and clinical disease in ducks infected with DAsTV-1 and -2 are poorly understood; therefore,
hindering differentiation with other viral hepatic diseases and limiting the ability to establish an
efficient diagnostic aid or practical vaccine. Recent research on astroviruses in poultry primarily
focuses on the identification and phylogenetic analyses for the purposes of understanding the
ecology and evolution. Therefore, additional work is necessary to understand the implications for
disease development and pathogenesis. Given the recently reported variability of astroviruses in
poultry and the potential for cross-species infection and evolution, future research must aim to
focus on the significance of each individual virus as well as co-infection potential in these commercial poultry species.

**Fowl Adenoviruses (FAdV)**

*Classification:* Inclusion Body Hepatitis (IBH) is caused by fowl adenoviruses (FAdV) of the genus *Aviadenovirus* (Group I avian adenoviruses) (114). Group II avian adenoviruses are the hemorrhagic enteritis (HE) virus of turkeys and marble spleen disease (MSD) virus of pheasants, both of which belong to the genus *Siadenovirus*. Duck adenovirus A causing egg drop syndrome (EDS) in laying hens is a member of the genus *Atadenovirus* and designated as Group III avian adenovirus. Within the genus *Aviadenovirus*, six species (FAdV A – E and goose adenovirus) and three tentative species (duck adenovirus B, pigeon adenovirus and turkey adenovirus B) are recognized (114). Twelve serotypes can be distinguished within FAdV A-E (115, 116). FAdVs causing IBH have predominantly been typed as fowl adenovirus D or E (115, 117).

*Transmission and prevalence:* All FAdVs including those responsible for IBH are assumed to transmit similarly. Horizontal transmission occurs mainly by the fecal-oral route (118). The virus is shed in the feces for up to six weeks (119). Adenoviruses are very resistant in the environment, and therefore fomites contaminated with feces are important in spreading virus. Airborne transmission is probable, since the virus can be re-isolated from the trachea and experimental intratracheal infection or infection by eye drop was successful (119). Vertical transmission is an important route of infection. Infected breeder hens shed virus in their eggs, thus infecting their progeny, as early as one week after experimental infection for at least five weeks (120).
Several serotypes can be detected sequentially during the live time of a flock or at the same time without necessarily be correlated to disease (121, 122). In Canada, approximately 77% of 231 randomly selected broiler flocks had been exposed to FAdV, with approximately 39% positive for FAdV D or E viral DNA (123). Outbreaks of IBH are often epidemic, such as those in the early 1970s in England (124) and Canada (125), and more recently in Korea and Japan (126, 127).

**Clinical signs and pathobiology:** The clinical course of IBH varies depending on the virulence of the virus, the age of the infected chickens and the presence of other pathogens, especially the immunosuppressive Chicken Infectious Anemia Virus (CIAV) and Infectious Bursal Disease Virus (IBDV).

IBH has been observed in vertically infected chickens as young as five days with mortality up to 5% and was experimentally reproduced in one day old SPF chickens who subsequently developed signs of the disease three days later (128). On the other hand, FAdV was repeatedly isolated from layers and broiler breeders of up to 45 weeks of age having IBH (129). Generally, older chickens seem to be less susceptible than younger ones. Mortalities after experimental infection of 2 days old chickens were between 50% and 83%, depending on the infective dose and route of infection, but only between 0% and 43% in 2 weeks old chickens (130). Co-infections with IBDV or CIAV aggravate the disease, and it was even doubted that FAdV were primary pathogens for IBH during co-infection (131). However, a high number of IBH cases was observed in New Zealand and Asia without the presence of IBDV and CIAV, and there was no correlation between cases of IBH or FAdV antibodies, respectively, with antibodies against IBDV and CIAV in Canada and Belgium (123, 132).

Clinical signs of IBH are nonspecific; chickens are depressed with ruffled feathers and having watery droppings (133). After experimental oral or ocular infection of chickens, deaths
were observed between five and twelve days post-infection with varying mortality (128, 134). Hepatic lesions are characterized by diffusely swollen, friable and pale liver (Fig. 4), sometimes with necrotic or hemorrhagic foci (Fig. 5) (127, 128, 135). Jaundice, especially in recovering birds, has also been described. Histological lesions are characterized by multifocal necrosis of hepatocytes and acute hepatitis. The namesake intranuclear inclusion bodies are located in the nuclei of the hepatocytes (Fig. 6) (134, 136, 137). Lesions in other organs have rarely been described. The mechanism of pathogenesis of IBH is largely unknown.

**Cross species infection:** Hepatitis with inclusion bodies caused by adenoviruses has been described in pigeons, parrots, cockatiels, falcons as well as turkeys (138-141). However, in most cases with a few exceptions, it is unclear if these viruses were FAdV or other adenoviruses. An adenovirus isolated from various psittacine species all exhibiting hepatitis with inclusion bodies was shown to be similar to FAdV D (138).

**Diagnosis:** Diagnosis in acute cases is primarily based on pathological lesions with laboratory confirmation by detection of FAdV, which is also required for typing. Since FAdV are almost ubiquitous, mere detection of FAdV does not prove the causal relationship with observed lesions. FAdV can be isolated in embryonated chicken eggs as well as in cell culture (142, 143). Serological methods such as ELISAs, serum neutralization test (SNT) and AGPT using anti-FAdV antisera can detect either all FAdV or only specific serotypes, depending on the test (144, 145). Numerous PCR assays have been used to detect FAdV followed by typing (146, 147). Liver samples generally yielded more positive results than fecal samples for the PCR assays.

**Prevention and control:** Autogenous vaccines against IBH-causing FAdV are used in some countries. Furthermore, an inactivated vaccine against FAdV is commercially available in China, and a live vaccine is available in Australia. Since IBH is of most concern in younger
broilers, and maternal antibodies are capable to protect progeny against IBH, broiler breeders are vaccinated with inactivated FAdV to induce maternal antibodies, which are then passed on to their progeny (133). In Australia, a live FAdV E vaccine is used to control IBH by vaccinating parent flocks in order to protect the progeny since 1989 (148). However, in spite of the vaccination, there are still sporadic outbreaks of IBH in Australia.

**Unanswered questions and future perspectives:** IBH is an old disease, but the increasing number of reported cases of IBH during the last five years indicates a need for better means to control the disease. Currently there are very good tools in place to diagnose the disease and type the causative viruses. However, there is a lack of understanding of the pathogenesis and of virulence factors of the virus. Therefore, future research is necessary to delineate the basis of viral pathogenesis and understand the structural and functional relationship of viral genes in order to develop better vaccines.

**Turkey Hepatitis Virus (THV)**

**Classification:** For a long time, a picornavirus had been suspected as possible causative agent for Turkey Viral Hepatitis (TVH) (149-151). In 2011, a novel picornavirus was identified by pyrosequencing as the likely causative agent of TVH and tentatively named Turkey Hepatitis Virus (THV) (152). Two strains of the novel picornavirus were identified with a 96.8% amino acid and 89.9% nucleotide sequence identities to each other. Phylogenetic analyses revealed that the novel picornavirus is distinct from other known picornaviruses, including Avian Encephalomyelitis Virus (AEV) (152).

**Transmission:** Experimental infections using the oral, intravenous, intraperitoneal, intraocular, and intratracheal routes were successful (149, 151-153). Since the virus is shed in the
feces and very resistant to inactivation, it can be assumed that the fecal-oral route is the main route of transmission. Since AEV is transmitted vertically, it is assumed that THV may also transmit vertically, although experimental evidence of vertical transmission is still lacking thus far (154). The disease seems to be most prevalent in winter, presumably due to the fact that cold weather favors the survival of the virus in the environment (154).

**Clinical signs and pathobiology:** Eleven of 17 THV cases in turkey flocks had been diagnosed in flocks younger than five weeks, and only two in flocks of 6-8 weeks of age (155). In a more recent study, TVH was observed in turkey flocks between 7 and 61 days old, with a median age of 28 days (154). Experimentally, the same virus inoculum caused the disease in two days old turkeys but not in four weeks old turkeys, suggesting potential age resistance against THV (150). In field cases, however, there was no correlation between the age of diseased flocks and the presence of gross lesions in the liver, and also the disease was experimentally reproduced in turkeys as old as eleven weeks in one study (154, 155). Under experimental conditions, clinical signs or mortality are lacking in infected birds, even though the infected birds at necropsies had pathological lesions (149-151, 155). Under field conditions, mortality was reported to be 1-25% in the late 1950s and early 1960s (155), and 0.03-5% daily mortality in the 2000’s (154). Other diseases were frequently observed in addition to TVH.

Whitish or tan foci in livers of affected birds are typical TVH gross lesions (Fig, 7) at post mortem (149, 151, 155, 156). However, analysis of 76 cases, in which TVH was diagnosed mainly based on histopathological lesions, showed that whitish or tan foci were only present in 46% of cases. In 28% of the cases, gross lesions were not present in the liver, while in the rest of cases, dark, congested, enlarged or greenish livers were observed (154). Histopathological liver lesions include mostly coagulative necrosis of hepatocytes and different stages of inflammation.
characterized by an influx of mononuclear inflammatory cells primarily composed of lymphocytes, macrophages and a few plasma cells and heterophils. Sometimes in subacute or chronic cases multifocal hepatitis without necrosis is present. Other hepatic lesions which have been described include biliary hyperplasia and the presence of giant cells or syncytia as well as vacuolation of hepatocytes (149, 151, 154-156). In addition to liver lesions, pale patchy areas in the pancreas (Fig. 8) characterized by focal necrosis of acinar cells with different stages of inflammation were also observed. Gross lesions in the pancreas were observed in 28% of cases and histopathological lesions in 46% of cases (154). Virus was re-isolated from liver, bile, feces, kidney, spleen and blood of experimentally-infected birds. THV genome was detected by PCR in liver, pancreas, intestines, feces and serum of naturally-infected birds (152, 155). It has been speculated that, after fecal-oral transmission, THV initially replicates in the intestine, subsequently the birds become then viremic, and the virus eventually localizes in the liver and pancreas causing lesions in these organs (154).

**Diagnosis:** Until recently, diagnosis was based mainly on the identification of gross and characteristic histopathological lesions. The causative agent, assumed to be THV, can be isolated in the yolk sac of embryonated chicken eggs causing high mortality of the embryos. The virus does not multiply after inoculation into the allantoic sac (156). More recently, a PCR assay and in situ hybridization have been reported for the detection of THV (152). Transmission electron microscopy can also be used to detect the virus with a low sensitivity. Immunohistochemistry using convalescent sera from naturally infected turkeys can detect virus antigen in livers of diseased poults (152).

**Unanswered questions and future perspectives:** Currently there are more questions than answers about TVH, despite the fact that the disease was first described more than five decades
ago. While there is a strong evidence that THV is the causative agent of TVH, the Koch postulates have not been fulfilled yet. The pathogenesis is unknown, the described syncytia in the liver require further study as there does not seem to be a relationship between a picornavirus and syncytia yet. It remains to be explored if TVH is in any way related to poult enteritis. Since the signs of TVH are subtle and can be easily missed, it is unknown how prevalent TVH and THV are. Lesions in the liver are also of nonspecific etiology and can be easily confused with bacterial infections, Ascarid larval migration and occasionally with histomonosis. THV-specific PCR and serology would certainly aid in diagnosis. The identification of two different strains in California and of a related strain in Hungary, which was associated with enteric disease, also raises the possibility that variant strains or genotypes may exist. Only when these questions are answered, the economic impact of TVH can then be more accurately assessed.
References

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FIGURES

Figures 1 and 2:

Fig. 1. Enlarged and hemorrhagic liver in a 42-week-old broiler breeder due to avian hepatitis E virus infection. Fig. 2. Enlarged liver with petechiae in a six-day-old duckling due to DHAV-1 infection.

Figure 3:

Fig. 3. Histopathology of the liver with severe diffuse necrosis of hepatocytes and hemorrhage due to DHAV–1 infection.
**Figure 4:**

Fig. 4. Diffusely enlarged and pale liver due to Fowl Adenovirus-1 (IBH) in a broiler chicken.

**Figure 5:**

Fig. 5. One diffusely enlarged and pale liver with petechiae and another liver with pale foci of necrosis in a broiler chicken due to IBH.
Figure 6:

Fig. 6. Histopathology of liver with hepatocellular necrosis, inflammation and intranuclear inclusion bodies in a case of IBH.

Figure 7:

Fig. 7. Enlarged liver with pale foci of necrosis in a young turkey poult suffering from Turkey Viral Hepatitis.
Fig. 8. Pancreas with pale patches of necrosis in a turkey poult to TVH.
Chapter IV: Infection Dynamics of Hepatitis E Virus in Wild-Type and Immunoglobulin Heavy Chain Knockout JH (-/-) Gnotobiotic Piglets

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ABSTRACT

Hepatitis E virus (HEV), the causative agent of hepatitis E, is an important but incompletely understood pathogen causing high mortality during pregnancy and leading to chronic hepatitis in immunocompromised individuals. The underlying mechanisms leading to hepatic damage remain unknown; however, the humoral immune response is implicated. In this study, immunoglobulin (Ig) heavy chain JH (−/−) knockout gnotobiotic pigs were generated using CRISPR/Cas9 technology to deplete the B-lymphocyte population resulting in an inability to generate a humoral immune response to genotype 3 HEV infection. Compared to wild-type gnotobiotic piglets, the frequencies of B-lymphocytes in Ig heavy chain JH (−/−) knockouts were significantly lower, despite similar levels of other innate and adaptive T-lymphocyte cell populations. The dynamic of acute HEV infection was subsequently determined in heavy chain JH (−/−) knockout and wild-type gnotobiotic pigs. The data showed that wild-type piglets had higher viral RNA loads in feces and sera when compared to the JH (−/−) knockout pigs, suggesting that the Ig heavy chain JH (−/−) knockout in pigs actually decreased level of HEV replication. Both HEV-infected wild-type and JH (−/−) knockout gnotobiotic piglets developed more pronounced lymphoplasmacytic hepatitis and hepatocellular necrosis lesions than other studies with conventional pigs. The HEV-infected JH (−/−) knockout pigs also had significantly enlarged livers both grossly and as a ratio of liver/body weight when compared with PBS-inoculated groups. This novel gnotobiotic pig model will aid in future studies into HEV pathogenicity, an aspect which has thus far been difficult to reproduce in the available animal model systems.

Keywords: Hepatitis E virus (HEV); gnotobiotic pig; Ig heavy chain knockout; B cell depletion
IMPORTANCE

According to World Health Organization, approximately 20 million HEV infections occur annually, resulting in 3.3 million cases of hepatitis E and > 44,000 deaths. The lack of an efficient animal model that can mimic the full-spectrum of infection outcomes hinders our ability to delineate the mechanism of HEV pathogenesis. Here, we successfully generated immunoglobulin heavy chain $J_H$ (-/-) knockout gnotobiotic pigs using CRISPR/Cas9 technology, established a novel $J_H$ (-/-) knockout and wild-type gnotobiotic pig model for HEV, and systemically determined the dynamic of acute HEV infection in gnotobiotic pigs. It was demonstrated that knockout of the Ig heavy chain in pigs decreased the level of HEV replication. Infected wild-type and $J_H$ (-/-) knockout gnotobiotic piglets developed more pronounced HEV-specific lesions than other studies using conventional pigs, and the infected $J_H$ (-/-) knockout pigs had significantly enlarged livers. The availability of this novel model will facilitate future studies of HEV pathogenicity.
INTRODUCTION

Hepatitis E, caused by hepatitis E virus (HEV), is typically an acute icteric disease of worldwide importance. Transmission of HEV occurs by the fecal-oral route, which often results in large explosive waterborne outbreaks in developing countries and sporadic foodborne cases in industrialized countries including the United States (1-4). The mortality rate for HEV infection ranges from 0.4-2% in immunocompetent healthy individuals (5); however, infected pregnant women experience a significantly elevated level of mortality, up to 28-30% (6, 7). While hepatitis E is generally recognized as a self-limiting acute disease, immunosuppressed individuals such as solid organ transplant recipients (8), individuals with concurrent HIV infections (9), and patients with lymphoma or leukemia tend to progress to a state of chronicity (10) with cirrhotic disease and elevated mortality (11). The underlying mechanisms of disease severity and hepatic damage experienced by these populations are currently not understood, nor do we possess an adequate animal model for addressing the current knowledge gaps.

HEV is a single-stranded, positive-sense RNA virus classified in the family Hepeviridae (12), which consists of two genera: Orthohepevirus and Piscihepevirus. Within the genus Orthohepevirus, 4 species are recognized. Species Orthohepevirus A includes all HEV strains that are known to infect humans and numerous other mammalian species. At least 8 distinct genotypes have been identified thus far within species Orthohepevirus A: genotypes 1-4 are known to infect humans (1, 12, 13) with genotypes 1 and 2 affecting only humans whereas genotypes 3 and 4 affect humans and several other animal species such as domestic pigs (14), deer (15), and rabbits (16, 17). Genotype 5 and 6 HEVs infect wild boars (18), genotype 7 HEV infects dromedary camels (19) and possibly humans (20), and genotype 8 HEV infects the Bactrian camel (19). Species Orthohepevirus B consists of HEV strains that infect avian species (21-23), Orthohepevirus C
infects rodents (24, 25), and Orthohepevirus D infects bats (26). The genus Piscipehevirus includes the sole strain of HEV infecting cutthroat trout (27).

Pigs are a major animal reservoir for HEV and a major source of zoonotic infections in humans (4). As the natural host for genotypes 3 and 4 HEV infections in humans (13, 28), the pig model has been used to study HEV biology and cross-species infections (13, 29). However, the typical outbred conventional pig experimentally infected with HEV does not develop the level of pathogenicity and progression of disease seen in immunocompromised and pregnant populations (30). Infection with HEV in conventional pigs are in general clinically asymptomatic with only mild to moderate hepatic changes observed (31). The typical course of HEV infection includes fecal shedding of HEV RNA in infected individuals at 1 week post-infection (wpi), which can persist for up to 8 wpi with a peak in viral titer at approximately 4 wpi (32, 33), a viremic phase lasting 1-2 weeks, followed by clearance of the virus at 8-9 wpi with the development of IgG anti-HEV at 2-4 wpi (34). Replication of HEV occurs primarily in the gastrointestinal tract (31, 35) with only limited level of virus replication in hepatocytes. As a result, direct viral effects within hepatic tissue is limited. Consequently, the humoral immune response has long been thought to exacerbate the hepatic disease process as an immune-mediated event, leading to the development of the observed liver lesions (36). Likewise, in non-human primates (37, 38) and chickens (39) experimentally infected with HEV, hepatic lesions and alterations in serum levels of liver enzymes often correspond to the appearance of HEV antibodies, further suggesting that anti-HEV IgG may play a role in the development of hepatic lesions.

Here we report the successful establishment of an immunoglobulin (Ig) heavy chain knockout JH (-/-) gnotobiotic piglet model that better mimics the course of acute HEV infection observed in humans. The dynamic of acute HEV infection was systematically determined in both
Ig heavy chain knockout and wild-type gnotobiotic piglets experimentally-infected with a genotype 3 human HEV. The presence and magnitude of viremia and fecal viral shedding, IgG anti-HEV antibody response to infection, immune correlates of infection, magnitude of infection and presence of viral RNA in extrahepatic sites, and liver pathology associated with HEV infection were determined.

**RESULTS**

**Successful development of Ig heavy chain JH (-/-) knockout gnotobiotic piglets.** In order to delineate the differentiating characteristics between Ig heavy chain JH (-/-) knockout and wild-type gnotobiotic piglets, CRISPR/Cas9 technology was utilized to alter the region designated as the Ig heavy chain leading to the generation of “JH (-/-) knockout” piglets (Table 1). Similarly, as demonstrated in our previous studies (40-42), no wild-type allele was observed in any of the genotyped embryos (Table 2 and Fig. 1), suggesting that the approach is effective in producing Ig heavy-chain knockout pigs. Pigs carrying the Ig heavy-chain knockout phenotype were produced by transferring CRISPR/Cas9 injected embryos into estrus matched surrogate sows. Included in this study was a total of 21 live piglets and one stillborn that were born from 6 surrogate dams (Tables 3 and 4). Genotyping results indicated that all Ig heavy-chain knockout pigs carried the modified Ig heavy chain region (Fig. 1 and Table 2). All wild-type gnotobiotic piglets were derived by embryo transfer of in vitro fertilized embryos carrying identical parental line as the Ig heavy-chain knockout pigs. Gnotobiotic piglets were retrieved by hysterectomy of pregnant sows with the removal of the entire uterus under sterile conditions and revived and housed in individual sterile isolators where all procedures occurred for the duration of the study.
Sterility of the surgical set-up, recovery chamber, and individual isolators was determined and confirmed by swabbing of the respective equipment and streaking on blood agar plates for any bacterial growth. The sterility of the gnotobiotic piglets was determined through the verification of a lack of bacterial growth (on both blood agar plates and Luria broth) from fecal swab samples throughout the course of the experiment. The JH (-/-) Ig heavy chain knockout gnotobiotic piglets were characterized and exhibited significantly reduced the numbers of CD79a⁺ B-lymphocytes (Fig. 2A and 2B) in the peripheral blood mononuclear cell (PBMC) populations, despite having normal levels of CD3⁺CD4⁺ lymphocytes and no alteration in CD16⁺ natural killer cell populations.

**Lower fecal viral RNA loads in HEV-infected JH (-/-) gnotobiotic piglets than in wild-type piglets.** In general, HEV infected conventional pigs shed virus in the feces by 1 week post-inoculation (wpi) with peak fecal viral shedding at 4 wpi and virus clearance by 7-8 wpi (33). In the Ig JH (-/-) gnotobiotic piglets infected with US-2 HEV in this study, viral RNAs were detected in feces as early as 4 days post-inoculation (dpi) with all piglets testing positive by 7 dpi and remained positive through necropsy at 28 dpi (Table 5). Likewise, the wild-type gnotobiotic piglets had detectable viral RNA in the feces from 7 dpi with all piglets testing positive by 9 dpi (Table 5) and remained so until necropsy at 28 dpi.

Quantification of HEV RNA loads in fecal samples (three times per week), weekly serum samples, intestinal content, bile, and hepatic as well as extrahepatic tissues including thymus, duodenum, jejunum, ileum, large intestine, gall bladder, spleen, liver, brain and spinal cord, and mesenteric lymph node was determined by qRT-PCR. The infected Ig JH (-/-) gnotobiotic piglets reached peak viral RNA loads in the feces at 16 dpi, whereas the viral RNA loads in infected wild-
type gnotobiotic piglets increased until 23 dpi (Fig. 3A and 3C). Viral RNA loads in both groups tapered but remained positive throughout the experimental period in accordance with the typical course of HEV infection. Neither group experienced a significant decrease in viral RNA loads by the 4 wpi necropsy timepoint. The fecal viral RNA loads were significantly higher in the HEV-infected wild-type gnotobiotic pigs than the JH (-/-) knockout gnotobiotic pigs (p<0.05) (Fig. 3A and 3C) at 23 dpi. The infected wild-type pigs also had numerically higher amounts of fecal viral RNA loads at other time points compared to the JH (-/-) knockout pigs, although the difference was not statistically significant at other time points. Likewise, the viral RNA loads in the intestinal content were also higher in the infected wild-type piglets than JH (-/-) knockout pigs; although, the difference was not significant (Fig. 3B). Overall, the JH (-/-) knockout pigs exhibited a reduced level of fecal HEV RNA shedding in the first 4 wpi compared to wild-type pigs; however, a significant difference was observed only at 23 dpi due to individual pig variations in both groups (Fig. 3A and 3C).

Lower serum viral RNA loads and fewer incidence of viremia in HEV-infected JH (-/-) piglets compared to wild-type piglets under gnotobiotic condition. In the HEV-infected JH (-/-) knockout pigs, when tested by a nested RT-PCR assay, 4/6 pigs became viremic by 14 dpi and 3/6 remained positive at necropsy at 28 dpi (Table 5). However, when tested by a quantitative qRT-PCR assay, which is less sensitive than nested RT-PCR and has a detection limit of 400 copies/µL sample, only 2 piglets in the JH (-/-) knockout pig group were positive for HEV RNA in the serum and none were positive until 3 wpi (Fig. 3D). In the infected wild-type pig group, viremia was detected as early as at 7 dpi with 4/6 pigs being positive at 14 dpi and 3/6 pigs remaining positive at necropsy at 28 dpi (Table 5). When tested by the qRT-PCR assay for the sera from the infected
wild-type pig group, the results were similar with that obtained by the nested RT-PCR assay: 1/6 positive at 7 dpi (Fig. 3D) and 4/6 positive at 14 dpi and remained positive by 28 dpi. The infected wild-type pigs had numerically higher amount of viral RNA loads in the serum than the JH (−/−) knockout pigs, although the difference was not significant (Fig. 3D). Overall, the data suggests that the level of viral RNA loads in serum is reduced in the JH (−/−) knockout pigs with fewer positive piglets when compared to the wild-type pigs.

Both the IgM and IgG anti-HEV responses in the JH knock-out and wild-type pigs were analyzed by ELISA. Anti-HEV antibody responses are typically observed during acute HEV infections by approximately 4-5 wpi in humans and conventional pigs (5, 32, 33). In this study, seroconversion to HEV antibodies was not detected in gnotobiotic pigs by the necropsy time point at 28 dpi (data not shown).

**No difference in the amount of viral RNAs in extrahepatic tissues between HEV-infected JH (−/−) knockout pigs and wild-type pigs.** During the acute phase of HEV infection in pigs, viral RNA is typically detectable in a variety of hepatic and extrahepatic tissues due to the circulating virus in the blood during the viremic stage. A qRT-PCR assay was used to quantify the amount of HEV RNA in samples of liver and various extrahepatic tissues including thymus, duodenum, jejunum, ileum, large intestine, gall bladder, spleen, brain and spinal cord, and mesenteric lymph node. HEV RNA was present in all tissues for the majority of piglets in both groups except in thymus and mesenteric lymph node. All thymus samples had very low viral RNA titers in only 2/6 JH (−/−) knockout piglets and 0/6 wild-type pigs. Mesenteric lymph node samples were positive at low levels in 3/6 JH (−/−) knockout piglets, but in all wild-type piglets (Data not shown). In the bile sample, the amount of HEV RNA was significantly higher (P=0.0169) in wild-type pigs than in JH
(-/-) knockout pigs (Figure 3B); although, there was no difference in viral RNA loads in other extrahepatic tissues between the JH (-/-) knockout and wild-type groups (Data not shown).

**Elevation of serum level of liver enzyme \(\gamma\)-glutamyl transferase (GGT), but not other enzymes, in HEV-infected JH (-/-) knockout gnotobiotic pigs at 3 wpi.** The serum levels of liver enzymes including aspartate aminotransferase (AST) (Fig. 4A), total bilirubin (Fig. 4B), and alkaline phosphatase (Fig. 4C) were assessed and found to be similar among the mock-infected JH (-/-) knockout, HEV-infected JH (-/-) knockout, mock-infected wild-type, and HEV-infected wild-type piglets. Total bilirubin appeared to be different between HEV-infected JH (-/-) knockout pigs and mock-infected JH (-/-) knockout piglets at 2-4 wpi; however, the differences were not statistically significant (Fig. 4B). The serum level of \(\gamma\)-glutamyl transferase (GGT) were found to be statistically elevated at 3 wpi (Fig. 4D) in HEV-infected JH (-/-) knockout gnotobiotic pigs when compared to the JH (-/-) knockout PBS group. There were abnormally elevated levels of GGT (10-100 IU/L considered within normal limits), AST, (10-100 IU/L), and total bilirubin (<1.0 mg/dL) in serum samples from all groups at various timepoints. In addition, all serum samples for all piglets in all groups had elevated alkaline phosphatase (>100 IU/L) from 0 wpi to 4 wpi (36 days of age) (Fig. 4C).

**Histological liver lesions characterized by lymphoplasmacytic hepatitis and hepatocellular necrosis in both HEV-infected JH (-/-) knockout and wild-type pigs.** At necropsy at 28 dpi, the histological lesions in the liver of infected pigs were characterized as lymphoplasmacytic hepatitis and hepatocellular necrosis (Fig. 5A). The average lesion score of lymphoplasmacytic hepatitis in HEV-infected JH (-/-) knockout pigs was significantly higher (P=0.0111) than that in PBS-
inoculated JH (-/-) knockout pigs. Similarly, the average lymphoplasmacytic hepatitis lesion score was also higher (P < 0.05) in HEV-infected wild-type pigs than in PBS inoculated wild-type pigs (Fig. 5A). The hepatocellular necrosis lesion score in HEV-infected wild-type pigs was also significantly higher (P < 0.05) than that in PBS-inoculated wild-type pigs but there was not a difference in lymphoplasmacytic hepatitis or hepatocellular necrosis lesion score between HEV-infected wild-type pigs and HEV-infected JH (-/-) knockout pigs (Fig. 5A).

**Higher liver/body weight ratio in HEV-infected JH (-/-) knockout pigs compared to PBS-inoculated controls.** The liver and body weights of each pig were measured at necropsy and the liver/body weight ratio was calculated to determine if the livers were enlarged. The average liver/body weight ratio in HEV-infected JH (-/-) knockout pigs was significantly higher than that of the PBS-inoculated JH (-/-) knockout (P < 0.01) or wild-type pigs (p < 0.05) (Fig. 5B), indicating that the presence of hepatic lesions and inflammation in this group led to larger livers than in the PBS-inoculated group. There was no significant difference in liver/body weight ratio between the wild-type pig infected and mock-infected groups (Fig. 5B).

**IFN-γ- and IL-4-specific CD4+ T-cell responses were not significantly altered in HEV-infected JH (-/-) knockout piglets when compared to HEV-infected wild-type pigs and mock-infected groups at 28 dpi.** PBMCs from heparinized plasma and mononuclear cells (MNCs) from spleen and mesenteric lymph nodes were isolated and stimulated with HEV-specific recombinant capsid antigen followed by staining and flow cytometry analysis (Fig. 6). HEV-specific T-cell (Th1) responses were analyzed for the frequency of IFN-γ and IL-4 expression and compared between mock-infected JH (-/-) knockout, HEV-infected JH (-/-) knockout, mock-infected wild-
type, and HEV-infected wild-type groups. Despite an apparent numerically lower level of expression of IFN-γ (Fig. 6A) in the PBMC T-cell population for all groups, the observed difference was not statistically significant. Expression of IL-4 (Fig. 6B) appeared to be numerically elevated in both mock-infected wild-type and HEV-infected groups when compared with the mock-infected and HEV-infected JH (-/-) knockout groups, again the difference was not statistically significant. In addition, total CD3⁺CD4⁺ T-cells (Th1) in the spleen and mesenteric lymph node were significantly elevated in both the mock-infected and HEV-infected JH (-/-) knockout groups when compared with wild-type infected groups (P<0.01) (Fig. 6C). The difference was discernible between both JH (-/-) knockout pig groups and the wild-type infected group at p<0.01; while, the difference between JH (-/-) infected and wild-type infected was more significant at p<0.001.

**IL-10- and TGF-β-producing CD4⁺CD25⁺Foxp3⁺ and CD4⁺CD25⁻Foxp3⁺ T-reg cell populations were not significantly altered in HEV-infected JH (-/-) knockout piglets when compared to HEV-infected wild-type pigs and mock-infected groups at 28 dpi.** To examine the influence of HEV infection on the functionality of Treg-cell subsets, we determined the frequencies of IL-10 and TGF-β expression as intracellular cytokines in PBMCs from heparinized plasma and MNCs from both splenic and mesenteric lymph node preparations. The mean frequencies of CD4⁺CD25⁺ and CD4⁺CD25⁻ Treg cell subsets (Fig. 7A and 7B) were similar among all groups in blood, spleen, and mesenteric lymph node. Likewise, the total Foxp3⁺ Treg cells in blood, spleen, and mesenteric lymph node were not significantly altered between the mock-infected and HEV-infected wild-type or JH (-/-) knockout groups of piglets (Fig. 7B). There was no discernible difference in the frequency of TGF-β (Fig. 7C) expression in any of the
aforementioned groups. Likewise, the IL-10 expression (Fig. 7D) was not significantly altered based on pig phenotype (wild-type versus JH (/-) knockout) or infection status (HEV versus mock-infected). There was an overall lower level of expression of IL-10⁺ CD4⁺ CD25⁺ Treg cells in the blood versus MNCs isolated from spleen or lymph nodes but this difference was consistent in all four groups regardless of infection or pig phenotype status (Fig. 7D).

**DISCUSSION**

HEV is an important but understudied pathogen with the potential to cause significant mortality in immunocompromised populations such as organ transplant recipients (8), those with concurrent systemic immunosuppressive diseases such as lymphoma (10) or HIV/AIDS (43, 44), and in HEV-infected pregnant women who are burdened with a higher mortality rate reaching up to 28-30% (5). In this study, we successfully established a novel gnotobiotic pig model for HEV infection, and systematically determined the dynamics of acute HEV infection in wild-type and Ig heavy chain JH (/-) knockout gnotobiotic piglets. We also attempted to investigate the role of immunoglobulin heavy-chain JH in HEV pathogenesis and acute virus infection.

Pigs are the natural host for genotypes 3 and 4 HEVs (13, 45, 46), which cause the majority of sporadic, cluster and chronic cases of hepatitis E in humans mostly from industrialized countries (4, 43). As an animal model, aside from non-human primates, pigs match humans (47) more closely than any other animals in terms of physiological characteristics and immunological parameters and responses. The gnotobiotic piglet model is extremely attractive especially for studying viral pathogenicity and immune responses, since the animals are raised in isolated sterile conditions with no interference from other infectious agents. In this case, infection with HEV was the only experimental manipulation in the piglets; therefore, the obtained results are solely
attributable and specific to HEV. In addition, it has been shown that gnotobiotic piglets are more susceptible to virus infection (48, 49) and tend to develop more progressive disease with discernible lesions. Lacking maternal antibodies at the time of infection may allow for the increased pathogenesis of HEV and afford the opportunity to utilize a model that more closely matches symptomatic human infection. This is important in the study of HEV infection because the conventional pig model develops only mild-to-moderate hepatitis lesions and clears the infection asymptotically (33, 50).

The application of the CRISPR/Cas9 system to produce Ig JH (-/-) knockout piglets significantly impacts the production of large animal models for biomedical research and in this case, allowed for the identification and isolation of each arm of the immune system in response to HEV infection. The CRISPR/Cas9 system allows for effective site-specific genome modification or targeted modifications during embryogenesis; therefore, bypassing the need for somatic cell nuclear transfer (SCNT), which is associated with developmental defects in animal production. In a previous study, we generated RAG2/IL2RG double knockout pigs using direct injection of CRISPR/Cas9 system (41), thus eliminating the breeding step in generating these pigs. The efficiency of this approach was high enough (100%) to place these pigs into a human norovirus challenge study without genotyping. Similarly, in this HEV infection study, all JH (-/-) knockout genetically modified pigs were generated using direct injection of CRISPR/Cas9 into developing embryos without reversion to wild-type genotyping. In addition, none of the Ig heavy-chain knockout piglets carried a mosaic genotype, which can be a shortcoming of the direct injection of the CRISPR/Cas9 system. These results suggest that the CRISPR/Cas9 system is a valid approach to use for animal production without having to establish a breeding program or herd for such use. Large animal models, namely pigs, closely recapitulate the clinical signs of disease in human
patients (51). However, use of the pig model in biomedical research is limited due to the cost of housing, housing requirements, and relatively prolonged gestation period. Using CRISPR/Cas9 system in large animal production may help reduce these costs through increased efficiency as well as opening up the opportunity to develop specific knock-out models in order to assess various immunological parameters.

During the acute phase of HEV infection, fecal virus shedding is typically apparent by 1 wpi and continues for ~3-4 weeks (32, 33, 52). By this time, the development of anti-HEV IgG is able to clear the infection, usually resulting in an asymptomatic course of infection devoid of clinical signs in both immunocompetent humans and pigs. The viremic phase typically lasts 1-3 weeks (34) and the acute infection is considered peak at 4 wpi. In the present study, it was important to demonstrate that this novel gnotobiotic pig model mimics the typical course of HEV infection in humans as this is the first reported use of gnotobiotic piglets for the study of HEV. To evaluate the gross and microscopic lesions in the liver attributable to HEV infection, the infected animals had to be euthanized at 28 days post-infection at which time the pathological liver lesions usually peak. In all HEV-inoculated gnotobiotic piglets, HEV was detectable in the feces during the first wpi and remained positive with relatively high viral RNA levels until necropsy at 28 dpi (Table 5, Fig. 3A and 3C). Fecal virus shedding appeared much earlier at 2 dpi in JH (-/-) knockout gnotobiotic piglets than in wild-type piglets (7 dpi). Interestingly, the viral RNA loads in fecal samples, intestinal content collected at necropsy, and serum samples were generally higher with significant differences observed at 23 dpi in the HEV-infected wild-type gnotobiotic piglets when compared to the HEV-infected JH (-/-) knockout gnotobiotic piglets. Furthermore, the incidence of viremia and viral RNA loads in sera were also higher in HEV-infected wild-type piglets than in HEV infected JH (-/-) knockout gnotobiotic piglets. It is possible that the decreased level of HEV
replication in JH (-/-) knockout gnotobiotic piglets is attributable to the lack of Ig heavy chain in the infected pigs. Unfortunately, due to the limited scope of the study, which is to study the infection dynamic and pathogenicity during acute infection, the animal experiment was terminated during the time of peak pathological liver lesions prior to the appearance of anti-HEV antibodies in pigs, which typically occurs at 4-5 weeks post-infection. The lack of detectable anti-HEV antibodies in infected JH (-/-) knockout and wild-type pigs at all time points validates the gnotobiotic pig model in that there are no maternal effects carried over after birth since piglets were retrieved via hysterectomy. Specifically, these piglets were naïve to colostrum and were maintained on sterile whole cow’s milk. Future studies are warranted to extend the infection period beyond the 4-week acute infection in order to definitively determine the role that the Ig heavy chain plays in susceptibility to virus infection and clearance. It is likely that the differences in virus replication level observed during the 4 weeks post-infection between the HEV-infected JH (-/-) knockout and wild-type groups will be greatly exacerbated with a prolonged experimental period, thus highlighting the importance of the humoral immune response to HEV infection.

HEV infection in immunocompetent humans and outbred conventional pigs generally causes only mild-to moderate hepatic lesions without clinical disease (2). In this study, both wild-type and JH (-/-) knockout gnotobiotic piglets experimentally infected with HEV developed more pronounced lymphoplasmacytic hepatitis and hepatocellular necrosis lesions than other studies using conventional pigs (Fig. 5A). All HEV-infected gnotobiotic piglets recorded a histopathologic score of 2, which corresponds to 3-5 focal infiltrates per 10 liver lobules or a 3 corresponding with 6-10 focal infiltrates per 10 liver lobules (33). Pigs with an assessed score of 3 also had hepatocellular necrosis or apoptosis, which was indicative of HEV-specific liver lesions. In addition, the HEV-infected JH (-/-) knockout pigs had significantly enlarged livers both grossly
and as a ratio of liver/body weight (Fig. 5B) when compared with PBS-inoculated group. Therefore, it appears that the HEV-infected gnotobiotic pigs induced more severe histological and gross liver pathology and inflammation than the conventional pigs. This presented as expected since gnotobiotic pigs are more sensitive to virus infection and are considered as an excellent model for pathogenicity studies of viral infections (48, 49). Therefore, the gnotobiotic pig model may aid in future studies of HEV pathogenesis and disease, an aspect which has thus far been difficult to reproduce in the available animal model systems.

Serum levels of liver enzymes during HEV infections tend to elevate at the time of peak virus infection (30, 53, 54). In this study, we showed that the serum levels of liver enzymes, with the exception of GGT (Fig. 4A-D), were not good indicators for liver damage nor do they assess overall liver health in the HEV-infected gnotobiotic piglets. Alkaline phosphatase was dramatically, yet inconsequentially elevated (Fig. 4C) in these piglets and likely due to the chosen feed source as well as the young age of the gnotobiotic piglets. It is likely that this enzyme will return to normal values after approximately 60 days of age. Furthermore, feeding sterile boxed cow’s milk in order to retain their sterility have had an adverse effect on the alkaline phosphatase levels, rendering it an insignificant biomarker in the gnotobiotic piglet model (55). Total bilirubin (Fig. 4B) and AST (Fig. 4A) were observed to be at similar levels in both the PBS mock-infected and HEV-infected groups. All animals presented with elevated values, albeit with no discernible trend or significance. The serum level of the liver enzyme GGT (Fig. 4D) was significantly elevated in HEV-infected JH (-/-) knockout gnotobiotic pigs at 23 dpi, which may indicate liver damage in HEV-infected JH (-/-) knockout pigs, since the predominant source of GGT is the biliary epithelium, and significantly higher HEV RNA loads were observed in bile (P=0.0169) in wild-type versus JH (-/-) knockout pigs.
At necropsy, neither the Ig heavy chain J\(_H\) (-/-) knockout or wild-type piglets had developed the antibodies necessary for clearance of HEV infection. By infecting gnotobiotic piglets at an early age (8 days) and completing the study by 35 days of age, the piglets are less immunocompetent than conventional pigs, which may result in a reduced humoral immune response and play a role in the level of HEV replication and pathogenesis. However, gnotobiotic piglets have been widely used to study other enteric viruses such as rotavirus infection dynamics and respond with a strong antibody response following infection by 28 dpi (56, 57). Therefore, the lack of antibodies is less likely influenced by the age of piglet, rather the typical time course for HEV infection. It has long been suspected that liver damage during HEV infection is a result of an immune-mediated (especially antibody-mediated) event and not due to the result of direct virus replication in hepatocytes (37, 38). Studies in non-human primates (38) demonstrated a correlation between liver lesions, elevated levels of liver enzymes, and symptomatic hepatic disease with the appearance of anti-HEV antibodies. Therefore, a longer term gnotobiotic pig infection study is needed to determine the significance of IgG anti-HEV antibodies in control of virus infection and pathogenesis and to determine the immune response of gnotobiotic piglets infected with HEV at an early age. Unfortunately, gnotobiotic piglets do have a rather limited lifespan due to the diet (sterile milk) and housing (plastic sterile bubble) requirements. The presence of liver lesions in the current study prior to an antibody response suggests that the liver damage is not solely mediated by the humoral immune response and requires further validation.

Cytokines play important roles regulating the immune response during virus infection but are currently not well understood in the context of HEV infection. During the acute HEV infection (up to 28 dpi) in the gnotobiotic pigs, we systematically determined the Treg, Th1, and Th2 cytokine responses in PBMC and splenic and mesenteric lymph node MNC populations. When
gated on CD3^+, CD4^+ Th1 cell frequencies in flow cytometric analyses were significantly elevated in the mock-infected and HEV-infected Ig heavy chain J_H (-/-) knockout groups when compared with wild-type piglets (Fig. 6C). However, the IFNγ^+ Th cells, which are the critical effector memory T-cells (58) used in pigs to mount cell-mediated immune responses, were not significantly different between any of the groups. The frequency of expression of IFN-γ^+ T cells appeared to be numerically lower in PBMCs and to a lesser degree in lymph node MNCs in both J_H (-/-) knockout groups when compared to the wild-type pig groups (Fig. 6A), but not in the MNCs populations isolated from spleen. Although the difference was not significant, it points to a trend in a somewhat dampened cell-mediated immune response in J_H (-/-) knockout pigs when compared to the wild-type piglet. In addition to the Th responses, Treg cells are also important in maintaining the balance between clearing virus infection and minimizing immune-mediated pathology during infection (59, 60). In this study, there was no difference in Treg cell populations (CD25^+ or CD25^-) between mock-infected and HEV-infected groups (Fig. 7A and 7B), regardless of the phenotypes of the pigs. Similarly, the Treg specific cytokines TGF-β and IL-10, typically expressed as a functional determination of the Treg cell population (Fig. 7C and 7D), were not significantly different among any of the groups. Therefore, while Treg cells may play a role in viral clearance or mitigation of immune-mediated pathology, this was not evident during the first 28 dpi of HEV infection in gnotobiotic pigs.

Overall, the results from this study showed a weak cell-mediated immune response in both the J_H (-/-) knockout and wild-type pigs at the presumptive peak virus infection at 28 dpi. Since this was the first attempt at HEV infection of gnotobiotic piglets with a limited study scope focusing on acute virus infection, the full course of infection from virus inoculation to clearance has not been evaluated. We demonstrated that Ig J_H (-/-) knockout phenotype pigs exhibit a
reduction in the mean frequencies of B lymphocytes while maintaining other cell populations including T-cells and NK cells. However, this study did not reach a time point when the wild-type pigs produced HEV-specific antibodies, which is a limitation. Therefore, a prolonged follow-up study is needed to fully discern the differences in immune responses between these JH (-/-) knockout and wild-type animals. Nevertheless, a discernible difference was observed with respect to liver lesions in HEV-infected gnotobiotic groups, a difference in the liver size both grossly and as a ratio of liver/body weight in the HEV-infected JH (-/-) knockout group, and a significant difference in viral RNA loads in bile at 28 dpi. The HEV infection dynamic data in gnotobiotic piglets from this study will help design future experiments using gnotobiotic pig model. Clearly, the JH (-/-) knockout gnotobiotic pig model for HEV requires further refinement to determine if anti-HEV antibodies play a role in pathogenicity and virus clearance and the presence of liver damage prior to the onset of an antibody response requires investigation.

MATERIALS AND METHODS

Generation of Ig JH (-/-) knockout gnotobiotic pigs by CRISPR/Cas9 technology. To disrupt the Ig heavy chain, a total of four target sites were designed using a web-based program (http://www.crispr-cas.org/p/resources.html) (Fig. 1A and Table 6). To minimize potential off-targeting events, target sequences were blasted against the whole pig genome and those carrying 17 or more identities with other loci in the pig genome were excluded. The four sgRNAs, containing a target sequence and tracker RNA, were synthesized by in vitro transcription via MEGASHORTSCRIPT™ Kit (Ambion) using a double-stranded DNA as a template (IDT). Cas9 mRNA was generated as described in our previous studies (40-42).
Pig oocytes were obtained either from ovaries collected at a local abattoir or purchased commercially (DeSoto, Inc). To collect oocytes from ovaries, medium-sized follicles were aspirated using an 18-gauge needle attached to a 10 ml syringe. Cumulus cell-oocyte complexes (COC) were washed twice with pre-warmed TL-Hepes medium and COCs with a multiple layer of cumulus cells, stimulated to mature in vitro in a TCM-199 based maturation media containing 0.5 IU/ml FSH, 0.5 IU/ml LH, 0.82 mM cysteine, 3.02 mM glucose, 0.91 mM sodium pyruvate, and 10 ng/ml EGF for 42-44 hours at 38.5 °C and 5% CO2 incubator. After maturation, COCs were denuded by 0.1% hyaluronidase. Oocytes were then extruded and the first polar body was used for in vitro fertilization (IVF). Groups of 30 oocytes were placed in 50 µl droplets of IVF medium (modified Tris-buffered medium with 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl2, 11 mM glucose, 20 mM Tris, 2 mM caffeine, 5 mM sodium pyruvate, and 2 mg/ml BSA) and covered with mineral oil. Extended semen was washed with PBS at 720 rcf, 3 min thrice. After the wash, the sperm pellet was resuspended with mTBM media. Subsequently, 50 µl of diluted sperm (2.5 × 10^5 sperm/ml) was added into mTBM drops that contained oocytes. The gametes were co-incubated for 5 hours at 38.5 °C and 5% CO2. Presumable zygotes were washed thrice with Porcine Zygote Media 3 (PZM-3) (61) then placed in PZM-3 and incubated at 38.5 °C, 5% CO2, and 5% O2.

Two hours after IVF, sgRNAs and Cas9 mRNA were introduced into presumptive zygotes to disrupt Ig heavy chain via microinjection. Each zygote received 10 ng/µl of sgRNAs and 20 ng/µl of Cas9 mRNA by direct injection into the cytoplasm using a FemtoJet microinjector (Eppendorf, Hamburg, Germany). Microinjection was conducted in manipulation medium (TCM199 with 0.6 mM NaHCO3, 2.9 mM HEPES, 30 mM NaCl, 10 ng/ml gentamicin, and 3 mg/ml BSA) and covered with mineral oil on the heated stage of a Nikon inverted microscope.
(Nikon Corporation, Tokyo, Japan). Injected zygotes were washed with PZM-3, then cultured in PZM-3 until further analyses. For embryo transfer, embryos were cultured in PZM-3 in the presence of 10 ng/ml GM-CSF (62).

To confirm mutations of the Ig heavy chain region, PCR verification was performed using the primers listed in Table 6. Genomic DNA was extracted from an individual blastocyst by incubating blastocysts in the embryo lysis buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 8.5, 0.5% Nonidet P40, 0.5% Tween-20 and 200 µg/ml proteinase K) for 30 min at 65 °C, followed by 10 min at 95 °C. Genomic DNA was extracted from the tissue of each piglet using PureLink Genomic DNA kit (Thermo Fisher Scientific) following the manufacturer’s instructions. The predicted mutation sites were amplified by using Platinum Taq DNA Polymerase (Thermo Fisher Scientific). PCR was conducted using the following conditions: initial denature at 95 °C for 2 min, denature at 95 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min for 34 cycles, final extension at 72 °C for 5 min, and holding at 4 °C. The PCR products were either directly sequenced or inserted into a cloning vector (TOPO, Invitrogen) to confirm the mutations introduced by CRISPR/Cas9 system.

At day 5-6 post IVF, blastocysts and embryos carrying over 16 cells were transferred into surrogate gilts. The embryos were surgically transferred into the oviduct of the gilts. Pregnancy was determined by ultrasound examination at day 30-35 of gestation. Sows impregnated with wild-type or knock-out embryos, respectively, underwent hysterectomy at 111-113 days of gestation for the retrieval of gnotobiotic piglets. Piglets were further maintained in plastic sterile microbiological isolators (“bubbles”) for the duration of the study. All animals were delivered and housed at Virginia Polytechnic Institute and State University (Blacksburg, VA) in accordance with
the approved procedures of the Institutional Animal Care and Use Committee (IACUC). Twenty-one gnotobiotic piglets were used, corresponding with 6 litters (IACUC no. 13-127-CVM).

**Virus inoculum.** A genotype 3 strain of human HEV (US-2) originally isolated from a patient in the United States was used in this study (63). The infectious stock of the US-2 HEV was prepared as a 10% fecal suspension in 0.01M PBS using the feces of a pig experimentally infected with the US-2 strain of human HEV from a previous study (34). The fecal suspension was further purified to produce an endotoxin-free virus preparation using cellulose sulfate (JNC Corporation, Tokyo, Japan) as the binding substrate for column purification and elution into PBS. The genomic equivalent (GE) titer of the final viral stock was determined by HEV-specific quantitative qRT-PCR with a GE titer of \(~4.5 \times 10^6\) per mL of fecal suspension.

**Experimental infection of wild-type and JH (-/-) knockout gnotobiotic piglets with a genotype 3 strain of human HEV.** The experimental design for the HEV infection of gnotobiotic pigs is detailed in **Table 1**. The gnotobiotic piglets were housed in the sterile isolators for 7 days before virus inoculation. On day 8, a total of 6 JH (-/-) knockout gnotobiotic piglets were intravenously inoculated with approximately \(9 \times 10^6\) GE titer of US-2 HEV infectious stock, and 5 other JH (-/-) knockout piglets were similarly mock-inoculated with PBS (**Table 1**). As controls, a total of 5 wild-type gnotobiotic pigs were intravenously inoculated with approximately \(9 \times 10^6\) GE titer of US-2 HEV infectious stock, and another 4 wild-type gnotobiotic pigs were similarly inoculated with PBS buffer.

After inoculation, the gnotobiotic piglets were monitored daily for a total of 4 weeks post-infection (wpi). Blood samples were collected from each pig weekly, and fecal samples were
collected three times per week. At 4 wpi, all gnotobiotic piglets were humanely euthanized and necropsied. Blood samples were collected for serum and PBMC isolation prior to euthanasia. The total body and liver weights were measured. During necropsy, tissue samples including spinal cord, brain, lung, liver, kidney, spleen, duodenum, jejunum, ileum, large intestine, and mesenteric lymph node were collected from each pig. A portion of the tissue samples were fixed in 10% formalin to process for routine histological examination of pathological lesions, and another portion of the tissues samples were immediately frozen on dry ice and further stored at -80°C for detection and quantification of HEV RNA by qRT-PCR (Table 5). Additionally, samples of bile and intestinal content were also collected and stored at -80°C for the detection and quantification of HEV RNA by RT-PCR and qRT-PCR (Figure 3B). Furthermore, sections of spleen and mesenteric lymph node were steriley collected in RPMI-1640 medium and stored on wet ice for immediate isolation of MNCs.

**Histological examination of liver lesions.** Evaluation of gross and histological lesion were conducted by a board-certified veterinary pathologist in a blinded fashion. The formalin-fixed tissues were trimmed, and routinely processed for histological examination. The lesion scoring criteria were previously described as follows (33): score 0, no inflammation; score 1, 1-2 focal lymphoplasmacytic infiltrates per 10 hepatic lobules; score 2, 2-5 focal infiltrates/10 hepatic lobules; score 3, 6-10 focal infiltrates/10 lobules; score 4, >10 focal infiltrates/10 hepatic lobules (Fig. 5A).

**Detection of HEV RNA by a nested RT-PCR.** To detect HEV RNA in weekly fecal samples, total RNAs were extracted with TRIzol LS Reagent (Invitrogen) from 200μL of each 10% fecal
suspension. The total RNAs were resuspended in 30µL of DNase-, RNase-, and proteinase-free water (Eppendorf Inc.). Synthesis of cDNA and first-round PCR amplification were performed using Superscript III one-step reverse transcriptase PCR kit (Invitrogen) with primers specific for the US-2 strain of HEV: forward primer US202:1198F22 (5’-ATCGCCCTGACACTGTTCAATC-3’), reverse primer US202:2064R25 (5’-AGGAATTAATTAAGACTCCCGGGTT-3’). The cDNA synthesis was carried out at 55°C for 30 minutes followed by PCR amplification with an initial denaturing incubation at 94°C for 2 minutes and 40 cycles of denaturation at 94°C for 15 second, annealing at 55°C for 30 seconds, and extension at 68°C for 1 minute, with a final incubation at 68°C for 5 minutes. The second round nested RT-PCR was completed using Hi-fidelity Taq polymerase (Invitrogen) using 5µL of first round PCR product as the template and the same primers US202:1198F22 and US202:2064R25 in a reaction as described previously (25, 47, 64). The amplified PCR products were visualized by gel electrophoresis on 1% agarose in Tris-acetate-EDTA buffer (TAE, Thermo Fisher Scientific). An expected PCR product band of 895 bp was extracted and purified from the gel using QG buffer (Qiagen), and sequenced to verify the authenticity of the amplified product at the Genomic Sequencing Center at the Biocomplexity Institute of Virginia Tech.

**Quantification of HEV RNA by qRT-PCR.** The amounts of viral RNAs in weekly serum and fecal swab samples as well as in a panel of tissue samples collected at necropsy were quantified by qRT-PCR using HEV-specific primers and probe. Briefly, fecal swab materials and intestinal content were suspended in 10% sterile PBS (w/v). Serum and bile samples were diluted in 10% sterile PBS (v/v). One gram of tissue samples (liver, spleen, jejunum, and lymph node) were homogenized in 1mL of TRIzol LS Reagent (Invitrogen) to prepare a 10% tissue suspension.
Briefly, each one-gram sample was placed in 1mL of TRIzol for 5 min at room temperature, homogenized using individual sterile gentleMACS dissociator tubes (Miltenyi Biotec), and centrifuged at 4°C for 5 min. Samples were separated into 1.5 mL microcentrifuge tubes for the addition of 200 µL chloroform per 1mL sample. Samples were incubated at room temperature for 10 minutes, centrifuged at 12,000 rpm for 15 min at 4°C for phase separation and supernatants added to an equivalent amount of 70% ethanol. Total RNAs were extracted using the RNeasy micro-kit columns (Qiagen) from 10% serum, bile, fecal swab materials, intestinal contents, and tissue suspension in 70% ethanol using a standard Qiagen protocol. The amounts of HEV RNA were quantified using the SensiFAST Probe No-ROX One-Step kit (Bioline, USA, Inc.) with the forward primer (JVHEVF, 5’GGTGTTTCTGGGTGAC-3’), reverse primer (JVHEVR, 5’-AGGGGTGTGGATGAA-3’), and hybridization probe (JVHEVP, 5’-TGATTCTCAGCCCTTCGC-3’) following the protocol as described previously (65). The qRT-PCR assays were performed in a CFX96 real-time (RT) PCR machine (Bio-Rad Laboratories). The standard curve was produced with purified HEV genomic RNAs, and the thermal cycling conditions were as follows: 45°C for 10 min (reverse transcription), 95°C for 2 min (initial denaturation), followed by 95°C for 5 s (denaturation), and 60°C for 20 s (PCR amplification) for 40 cycles. The detection limit of the qRT-PCR assay was reported earlier as 10 viral genomic copies, which corresponds to approximately 400 copies of viral RNA per 1 mL sample or per gram tissue (65, 66). Samples with GE titers below the detection limit were considered negative.

**ELISA to detect IgG anti-HEV in pigs.** Weekly serum samples were tested for IgG anti-HEV antibodies by an enzyme-linked immunosorbent assay (ELISA) as described previously (14, 34, 47). Briefly, a truncated recombinant HEV capsid protein containing the immunodominant region
of HEV ORF2 (amino acids 452-617) was used as the antigen (GenWay Biotech Inc., San Diego, CA), and plated at 6 µg/10mL carbonate coating buffer to each well. The serum samples were tested at 1:100 dilution in blocking buffer. Pre-immune and hyperimmune anti-HEV positive pig sera were included as the negative and positive controls, respectively. Horse-radish peroxidase (HRP)-conjugated goat anti-swine IgG (Sigma) was used as the secondary antibody at 1:2,000 in blocking buffer. The plate was developed using ABTS substrate and absorbance was measured at 405 nm on a Promega GloMax Discover plate reader.

**Determination of serum levels of liver enzymes in pigs.** A panel of liver enzymes including aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase, and total bilirubin were measured in weekly serum samples using established protocols and standard values from the Veterinary Diagnostic Lab at Iowa State University College of Veterinary Medicine (Ames, IA).

**Isolation of PBMCs from peripheral blood and MNCs from spleen and lymph node tissues.** Blood was collected from each pig for PBMC isolation prior to euthanasia and isolated using density-gradient centrifugation with Ficoll-Paque PREMIUM (GE Healthcare). Buffy coats were resuspended in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Viability counts of PBMCs were conducted with 1:1 Trypan blue as the stain and a Cellometer (Nexelom). Cells were stored at a density of 1x10^7/mL in FBS with 5% DMSO at -80ºC overnight before moving to liquid nitrogen for storage prior to staining. Samples of spleen and MLN tissues were collected in RPMI-1640 medium supplemented with gentamicin, ampicillin, and HEPES on the day of euthanasia and processed for the isolation of
MNCs as described previously (67, 68). Briefly, the spleen tissues were minced and transferred through a 40 µm cell strainer and pelleted by centrifugation. A density-gradient isolation procedure was followed using Percoll in order to collect the interface containing MNCs. The isolated cells were resuspended in enriched RPMI-1640 medium supplemented with 8% FBS, gentamicin, ampicillin, 2 mM L-Glutamine, 100 µM non-essential amino acids, 1 mM sodium pyruvate, and 1:1000 2-mercaptoethanol. For the mesenteric lymph node (MLN), the tissue samples were incubated for 30 minutes at 37°C on a horizontal shaker in RPMI medium supplemented with 8% FBS, gentamicin, ampicillin, HEPES, collagenase D (1mg/mL), and DNase I (100U/mL), prior to transferring the suspension to a cell strainer and collection by centrifugation. Cells were counted with 1:1 Trypan blue using Cellometer and stored as described above.

**Intracellular cytokine staining and flow cytometry analyses.** Splenic and mesenteric lymph node MNCs and blood PBMCs were thawed at room temperature, resuspended in RPMI-1640 medium with 10% FBS, ampicillin, and gentamicin, and plated at a concentration of 2x10^6 cells/100µL/well of a 96-well V-bottom tissue-culture plate. Two wells were prepared for each sample and 3 separate plates for each of the 3 staining protocols. An unstimulated preparation of RPMI-1640 medium with 10% FBS, ampicillin, gentamicin, and 0.2 µg anti-human CD49 costimulatory mAb was applied to one well for each sample per plate at 100 µL per well. A stimulation preparation of RPMI-1640 medium with 10% FBS, ampicillin, gentamicin, 0.2 µg CD49 mAb, and 1µL JPT Pepmix (purified recombinant HEV ORF2 antigen) was applied to one well for each sample per plate at 100 µL per well. A cell stimulation mixture (eBioscience, Inc.) was used as a positive stimulation control. Cells were stimulated for 12 hr at 37°C. Brefeldin A (GolgiPlug, BD Biosciences) was added to each well at 0.2 µL/well and further incubated at 37°C
for 5 hr. Plates were stored overnight and protected from light at 4°C prior to antibody staining. PBMCs and MNCs were washed with PBS once, resuspended in cold PBS with 3% FBS (FACS buffer), and stained with one of the following three panels of fluorochrome-conjugated antibodies according to manufacturer’s recommendations and procedures described previously (45, 48, 49).

In panel one, PBMCs (2x10^6 cells per well) were sequentially stained with the following mAb sets diluted in cold PBS with 3% FBS at 4°C for 15 min per incubation period: Spectral Red-conjugated mouse (IgG1) anti-pig CD3ε (clone PPT3; Southern Biotech) and phycoerythrin (PE)-conjugated mouse (IgG1) anti-pig CD16 (AbDSerotec) followed by permeabilization with BD Cytofix/Cytoperm buffer (BD Pharmigen) at 4°C for 15 min and washing thrice with BD Perm/Wash buffer (BD Pharmigen). Intracellular markers were stained with mouse IgG2b anti-human CD79a (Clone 11D10, Vector Laboratories), followed by PE-Cy7-goat anti-mouse IgG2b (Southern Biotech) and FITC-conjugated rat anti-mouse (IgG2b) (BioLegend) at 4°C for 15 min. Appropriate isotype-matched control antibodies were included as background staining controls (Southern Biotech, BD Biosciences, eBioscience, and Affymetrix ebioscience). The frequencies of CD16^+ and CD79a^+ cells were expressed as percentages of parental CD3 gated cells.

In panel two, in vitro HEV-specific antigen-stimulated PBMCs and splenic and mesenteric lymph node MNCs were stained with antibodies to determine the frequencies of CD3^+CD4^+Foxp3^+ T reg cells, CD25^+ activation status, and the expression of regulatory cytokines IL-10 and TGF-β in these cells (45, 48). PBMCs, spleen-derived MNCs, and mesenteric lymph node-derived MNCs (2x10^6 cells/well) were stained at 4°C for 15 min with FITC-conjugated mouse (IgG2b) anti-pig CD4a, SPRD-conjugated mouse (IgG2a) anti-pig CD3, and mouse (IgG1) anti-pig CD25 (IgG1, clone K231.3B2; AbD Serotec), followed by APC-conjugated rat (IgG1) anti-mouse (clone X56, BD Pharmingen). After staining for the extracellular markers, the cells were permeabilized with
the Foxp3-staining buffer set (eBioscience) at 4°C for 15 min. Intracellular staining was completed with PE-Cy7-conjugated rat (IgG2) anti-mouse/rat Foxp3 (cloneFJK-16s; eBioscience), Biotin mouse (IgG1) anti-pig IL-10 (clone 945a; Cell Sciences), and PE-conjugated mouse (IgG1) anti-human TGF-β1 (clone 9016; R&D system) at 4°C for 15 min. The appropriated isotype-matched control antibodies and PE-conjugated mouse (IgG1) isotype control (clone P3.6.2.8.2; eBioscience) were used as background staining controls. The frequencies of CD4+Foxp3+, CD25+, IL-10+, and TGF-β+ cells were expressed as percentages of parental CD3 gated cells.

In panel three, in vitro HEV-specific antigen-stimulated PBMCs and spleen-derived and mesenteric lymph node-derived MNCs were stained with antibodies to determine CD3+CD4+ cells expressing IL-4 and IFN-γ regulatory cytokines. PBMCs and MNCs were stained at 4°C for 15 min with FITC-conjugated mouse (IgG2b) anti-pig CD4a (clone 74-12-4; BD Pharmingen) and SPRD-conjugated (IgG1) anti-pig CD3ε followed by permeabilization with BD Cytofix/Cytoperm buffer (BD Pharmingen) at 4°C for 15 min. PBMCs and MNCs were washed three times with BD Perm/Wash buffer (BD Pharmingen) and subsequently stained with PE-conjugated mouse (IgG1) anti-pig IFN-γ (clone P2G10; BD Pharmingen) and Brilliant Violet 421 anti-human IL-4 antibody (IgG1 clone MP4-25D2; BioLegend). The appropriate isotype-matched control antibodies were used as background staining controls (BioLegend, BD Biosciences, and Southern Biotech).

For all three staining panels, at least 100,000 events were acquired on FACS Calibur flow cytometer (BD Biosciences) and the data were analyzed using FloJo V10 software (Tree Star). The absolute number of B, Treg, and NK-cells were based on the frequencies of cells in each sample, respectively.
**Statistical analyses.** All data was statistically analyzed using GraphPad Prism 7.0 (GraphPad Software Inc.). The differences between the mean values of two treatment groups were analyzed with two-way ANOVA or two-tailed unpaired student’s t-test and Tukey multiple comparison tools. The presence or absence of hepatic infiltrates was analyzed by Fisher’s exact test. A p-value of <0.05 was considered statistically significant for all analyses. All graphs were reported as the mean +/- SEM.

**ACKNOWLEDGMENTS**

This study is supported by grants from the National Institutes of Health (R01 AI074667, and R01 AI050611). Danielle M. Yugo is supported by a Ruth L. Kirschstein National Research Service Award Institutional Research Training Grant (T32OD010430). We thank Ms. Melissa Makris for her technical assistance in flow cytometry, Karen Hall, Kimberly Allen, Peter Jobst, and the Teaching and Research Animal Care Support Staff (TRACCS) for their support in the animal study, and Dr. Lijuan Yuan’s laboratory for their support in the animal study.
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TABLE 1. Experimental infection of wild-type and immunoglobulin J\(_H\) (-/-) knockout gnotobiotic piglets with a genotype 3 human strain (US2) of hepatitis E virus

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Piglets(^a)</th>
<th>Phenotype of Piglets</th>
<th>Inoculum(^b)</th>
<th>Necropsy at 4 wpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>J(_H) Knock-out</td>
<td>PBS</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>J(_H) Knock-out</td>
<td>G3 (US2) HEV</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>Wild-type</td>
<td>PBS</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>Wild-type</td>
<td>G3 (US2) HEV</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^a\) Piglets were retrieved by hysterectomy at 111-113 days of gestation and maintained germ-free in sterile isolators for the duration of the study. Variation in piglet numbers per groups was due to the efficiency in retrieval of live piglets following embryo transfer.

\(^b\) Piglets were inoculated with US2 strain of HEV or mock (PBS) at 8 days of age via ear-vein injection.
TABLE 2. Efficacy of CRISPR/Cas9 to introduce targeted modifications in Ig heavy chain locus during embryogenesis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Total # injected</th>
<th>% of blastocysts on day 7</th>
<th># of genotyped</th>
<th>Genotypes&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Homozygous</td>
</tr>
<tr>
<td>Ig heavy chain</td>
<td>132</td>
<td>24 (18.2%)</td>
<td>15</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Single embryos at blastocyst stage were used for genotyping. No wild-type allele was found.
TABLE 3. Phenotyping of Ig heavy-chain modified piglets produced using CRISPR/Cas9 technology and retrieved by hysterectomy

<table>
<thead>
<tr>
<th>Surrogate ID</th>
<th>Modification</th>
<th>Number of embryos transferred into a surrogate</th>
<th>Stage of embryo</th>
<th>Pregnancy</th>
<th>Number of pigletsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>308</td>
<td>Ig Heavy chain</td>
<td>82</td>
<td>Day 6</td>
<td>Y</td>
<td>3 piglets and 1 mummy</td>
</tr>
<tr>
<td>68-8</td>
<td>Ig Heavy chain</td>
<td>74</td>
<td>Day 6</td>
<td>Y</td>
<td>5 piglets</td>
</tr>
<tr>
<td>61-12</td>
<td>Ig Heavy chain</td>
<td>89</td>
<td>Day 5</td>
<td>Y</td>
<td>4 piglets</td>
</tr>
<tr>
<td>29-1</td>
<td>Ig Heavy chain</td>
<td>81</td>
<td>Day 6</td>
<td>Y</td>
<td>3 piglets</td>
</tr>
<tr>
<td>61-13</td>
<td>Ig Heavy chain</td>
<td>80</td>
<td>Day 6</td>
<td>Y</td>
<td>4 piglets</td>
</tr>
<tr>
<td>Y465</td>
<td>WT</td>
<td>83</td>
<td>Day 6</td>
<td>Y</td>
<td>8 piglets and 3 mummy</td>
</tr>
<tr>
<td>70-3</td>
<td>WT</td>
<td>89</td>
<td>Day 5,6</td>
<td>Y</td>
<td>8 piglets</td>
</tr>
<tr>
<td>55-09</td>
<td>WT</td>
<td>68</td>
<td>Day 6,7</td>
<td>Y</td>
<td>2 piglets</td>
</tr>
<tr>
<td>70-04</td>
<td>WT</td>
<td>105</td>
<td>Day 5</td>
<td>Y</td>
<td>2 piglets</td>
</tr>
</tbody>
</table>

aA total of 21 piglets were delivered from 5 surrogate gilts carrying the immunoglobulin heavy chain knockout JH (-/-) phenotype. The piglets were maintained in gnotobiotic status in sterile isolators for the duration of the experimental period.
TABLE 4. Genotyping the Ig heavy chain modified piglets

<table>
<thead>
<tr>
<th>Pig ID</th>
<th>Mutation</th>
<th>Genotype $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>N/A</td>
<td>GCACACCCCCCAGGTTTTTTGTTGGGGCGAGCTGGAGATTGCAACACT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>308-1</td>
<td>Homozygous</td>
<td>GCACACCCCCCCAGGTTTTTTAGTGGGGCGAGGCCT (1bp)</td>
</tr>
<tr>
<td>308-2</td>
<td>Biallelic mutation</td>
<td>GCACACCCCCCAGGTTTTTTGTGGGGCGAGCTGGAG (1bp)</td>
</tr>
<tr>
<td>308-3</td>
<td>Homozygous</td>
<td>GCACACCCCCCAGGTTTTTTAGTGGGGCGAGGCCT (4bp)</td>
</tr>
<tr>
<td>68-8-1</td>
<td>Biallelic mutation</td>
<td>GCACACCCCCCAGGTTTTTTGTGGGGCGAGCTGGAG (4bp)</td>
</tr>
<tr>
<td>68-8-2</td>
<td>Homozygous</td>
<td>GCACACCCCCCAGGTTTTTTGTGGGGCGAGCTGGAG (4bp)</td>
</tr>
<tr>
<td>68-8-3</td>
<td>Biallelic mutation</td>
<td>GCACACCCCCCAGGTTTTTTGTGGGGCGAGCTGGAG (3bp)</td>
</tr>
<tr>
<td>68-8-4</td>
<td>Biallelic mutation</td>
<td>GCACACCCCCCAGGTTTTTTGTGGGGCGAGCTGGAG (5bp)</td>
</tr>
<tr>
<td>68-8-5</td>
<td>Biallelic mutation</td>
<td>GCACACCCCCCAGGTTTTTTGTGGGGCGAGCTGGAG (1bp)</td>
</tr>
<tr>
<td>61-12-1</td>
<td>Homozygous</td>
<td>GCACACCCCCCAGGACACCTCCCCCTCCAAGGGCGAGCTGGAG (14bp)</td>
</tr>
<tr>
<td>61-12-2</td>
<td>Homozygous</td>
<td>TCCCAGCCTCCGCGCGAGG---GTGGGGCGAGCTGGAG (59bp)</td>
</tr>
<tr>
<td>Piglet</td>
<td>Homozygous</td>
<td>Biallelic Mutation</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>61-12-3</td>
<td>Homozygous</td>
<td>GCACACCCCCCAGG------GGGGCGAGCCTGGAG</td>
</tr>
<tr>
<td>61-12-4</td>
<td>Biallelic mutation</td>
<td>GCACACCCCCCCAGGGTTTTTTGTGGGGCGAGCCTGGAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGGAAAGTCAGAGGGAGGAGC-------AGATTGCACCAC</td>
</tr>
<tr>
<td>29-1-1</td>
<td>Homozygous</td>
<td>GCACACCCCCCAGGT----GTGGGGCGACCT</td>
</tr>
<tr>
<td>29-1-2</td>
<td>Homozygous</td>
<td>GCACACCCCCCA------GGGGCGAGCCT</td>
</tr>
<tr>
<td>29-1-3</td>
<td>Biallelic mutation</td>
<td>GCACACCCCCCA------GGGGCGAGCCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCACACCCCCCAAGGTTTTTTTTTTTTGGGGCGAGCCT</td>
</tr>
<tr>
<td>61-13-1</td>
<td>Biallelic mutation</td>
<td>GCACACCCCCCCCCAGGT----GTGGGGCGAGCCTGGAGATTGCACCAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTGATTACTATATGCTATGGATCTCTGTGGGGCGAGGCTGAGTCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCGTGCTCTGTAAGACGGCCC</td>
</tr>
<tr>
<td>61-13-2</td>
<td>Biallelic mutation</td>
<td>GCACACCCCCCAGGTTTGTGT----TCGTGTCTCTAGGTAAGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CGGCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCACACCCCC----CAGGTAAGACGGCCC</td>
</tr>
<tr>
<td>61-13-3</td>
<td>Homozygous</td>
<td>GCACACCCCCCAGGTTTTGTGT----CGTGCTCTCAGGT</td>
</tr>
<tr>
<td>61-13-4</td>
<td>Homozygous</td>
<td>GCACACCCCCCAGGTTTTTTTT----CTCAGGT</td>
</tr>
</tbody>
</table>

“Genotype of each knock-out piglet with mutations on each allele individually or on both alleles. Number of base pairs for each indicated in parentheses. **Underline** indicates targeting sequence. **Bold letters** indicate insertion or change in nucleotides, and ‘-‘ indicates deletion of a nucleotide. All piglets carried mutation on Ig heavy chain.
TABLE 5. Detection of HEV RNA in the serum (fecal) samples of wild-type and Ig J<sub>H</sub> (<i>-/-</i>) knockout gnotobiotic piglets experimentally infected with the US2 strain of human HEV

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum</th>
<th>Piglet phenotype</th>
<th>No. of positive samples/no. tested on DPI&lt;sup&gt;a&lt;/sup&gt;:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>PBS</td>
<td>Knock-out</td>
<td>0/5 (0/5)</td>
</tr>
<tr>
<td>2</td>
<td>HEV</td>
<td>Knock-out</td>
<td>0/6 (0/6)</td>
</tr>
<tr>
<td>3</td>
<td>PBS</td>
<td>Wild-type</td>
<td>0/4 (0/4)</td>
</tr>
<tr>
<td>4</td>
<td>HEV</td>
<td>Wild-type</td>
<td>0/6 (0/6)</td>
</tr>
</tbody>
</table>

<sup>a</sup>DPI: days post-inoculation. HEV RNA was detected via strain-specific nested RT-PCR and qPCR. The amplified PCR-positive products were sequenced to confirm the identity as the US2 strain of HEV.
TABLE 6. Primers and double-stranded DNA (dsDNA) used for the development of Ig heavy-chain knockout piglets

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ig Heavy chain G-block 1</td>
<td>TTAATACGACTCACTATAGGGTGAGTCGTCTGTC GCTTTTAGAGCTAGAAATAGCAAGTTAAATTAGCTA</td>
</tr>
<tr>
<td>(dsDNA)</td>
<td>GTCCGGTTATCAACTTGGAAAGTGGCAGCCGAGTCGGTG CTTTTTTGTTTTAGA</td>
</tr>
<tr>
<td>Ig Heavy chain G-block 2</td>
<td>TTAATACGACTCACTATAGGGACACCCCCAGAGTTTTTG TGGTTTTAGAGCTAGAAATAGCAAGTTAAATTAGCT</td>
</tr>
<tr>
<td>(dsDNA)</td>
<td>AGTCGGTTATCAACTTGGAAAGTGGCAGCCGAGTCGGTG CTTTTTTGTTTTAGA</td>
</tr>
<tr>
<td>Ig Heavy chain G-block 3</td>
<td>TTAATACGACTCACTATAGGGACACCCCCAGGTTTTTG TGGTTTTAGAGCTAGAAATAGCAAGTTAAATTAGCT</td>
</tr>
<tr>
<td>(dsDNA)</td>
<td>AGTCGGTTATCAACTTGGAAAGTGGCAGCCGAGTCGGTG CTTTTTTGTTTTAGA</td>
</tr>
<tr>
<td>Ig Heavy chain G-block 4</td>
<td>TTAATACGACTCACTATAGGGACACCCCCAGGTTTTTG TGGTTTTAGAGCTAGAAATAGCAAGTTAAATTAGCT</td>
</tr>
<tr>
<td>(dsDNA)</td>
<td>AGTCGGTTATCAACTTGGAAAGTGGCAGCCGAGTCGGTG CTTTTTTGTTTTAGA</td>
</tr>
<tr>
<td>T7_Ig Heavy chain 1F</td>
<td>TTAATACGACTCACTATAGGGTGAGTCGTCTGTC</td>
</tr>
<tr>
<td>T7_Ig Heavy chain 2F</td>
<td>TTAATACGACTCACTATAGGGACACCCCCAGAGTTTTTG TG</td>
</tr>
<tr>
<td>T7_Ig Heavy chain 3F</td>
<td>TTAATACGACTCACTATAGGGAGATTGCAGATTGGCAGCCGAGTCGGTG TGGTTTTAGAGCTAGAAATAGC</td>
</tr>
<tr>
<td>T7_Ig Heavy chain 4F</td>
<td>TTAATACGACTCACTATAGGGTGAGTCGTCTGTC</td>
</tr>
<tr>
<td>T7_sgRNA R</td>
<td>AAAAGCCACCGACCTCGGTGCC</td>
</tr>
<tr>
<td>Cas9 F</td>
<td>TTAATACGACTCACTATAGGGAGATGGGACTATAAGGA CCACGAC</td>
</tr>
<tr>
<td>Cas9 R</td>
<td>GCGAGCTCTAGGAATCTCTTCAC</td>
</tr>
<tr>
<td>Genomic Primer F</td>
<td>GACACTTTGGAGGTACAGGAGAAGGACTATAAGGA</td>
</tr>
<tr>
<td>Genomic Primer R</td>
<td>CTTCTCTCTCCGACATGGCTCTTTTGCAGAC</td>
</tr>
</tbody>
</table>
Fig. 1. Ig heavy chain targeting CRISPR sequences and representative types of Ig heavy chain mutations in this study. (A) Disruption of Ig heavy chain in pigs by CRISPR/Cas9 with a total of 4 target sites designed using a web-based program. Bold letters indicate PAM sequence of target sites. Black arrows indicate primers used to amplify the target region. (B) Types of genetic mutations of the Ig heavy chain in single blastocyst. The sequencing results indicated either homozygous or biallelic mutation of Ig heavy chain in single embryos with no wild-type alleles observed. (C) Representative genotype of Ig heavy chain knock-out pigs. Arrows indicate sites of mutations.
Fig. 2. B-lymphocyte cell counts in peripheral blood samples of wild-type and immunoglobulin J_{H} knockout gnotobiotic piglets experimentally-infected with HEV. PBMCs were collected from each piglet at 28 days post-inoculation (dpi) at necropsy. Cells were gated based on CD3 and quantified for the intracellular marker CD79a^{+} as a measure of the total B-cell population in the peripheral blood. Asterisks indicate statistical significance between designated groups determined by Tukey’s t-test with a p value <0.001.
Fig. 3. Quantification of HEV RNA loads in serum, fecal, and bile samples of wild-type and JH knockout gnotobiotic piglets experimentally-infected with HEV. Viral RNAs were extracted from (A) fecal samples three times weekly, (B) intestinal contents and bile at 28 days post-inoculation (dpi), (C) as a scatterplot of fecal viral RNA, with each symbol indicating the value for an individual piglet, and (D) serum samples at 0, 7, 14, 21, and 28 dpi for the quantification of HEV RNA copy numbers by qRT-PCR. Data are expressed as mean +/- SEM. The detection limit is 10 viral genomic copies, which corresponds to 400 copies per 1mL sample or per gram of tissue. Titers below the reported detection limit were considered negative. Asterisks indicate statistical significance between designated groups as determined by two-way ANOVA and p-value <0.05.
Fig. 4. Circulating levels of liver enzymes in serum samples collected weekly from HEV-infected wild-type and HEV-infected J_H (-/-) knockout gnotobiotic piglets. Serum was collected weekly from each piglet beginning with 0-week post-inoculation (wpi) and continuing until necropsy at 4 wpi. Liver enzymes including (A) aspartate aminotransferase (AST), (B) total bilirubin, (C) alkaline phosphatase, and (D) gamma-glutamyl transferase (GGT) were measured at the Iowa State University Veterinary Diagnostic Lab. Normal limits were provided with the analysis based on the laboratory’s standards for each specific test, which are indicated on each scatterplot graph as a dotted line. Each sample indicates an individual piglet for each time point and are expressed as the mean +/- SEM. Analysis was completed using two-way ANOVA. *p<0.05.
Fig. 5. Histopathological lesions in wild-type and JH knockout gnotobiotic piglets experimentally-infected with HEV. (A) Histopathological lesions in the liver including lymphoplasmacytic hepatitis and hepatocellular necrosis. Lymphoplasmacytic hepatitis was scored as follows: 0, no inflammation; 1, 1-2 focal lymphoplasmacytic infiltrates/10 hepatic lobules; 2, 3-5 focal infiltrates/10 hepatic lobules; 3, 6-10 focal infiltrates/10 hepatic lobules; and 4, >10 focal infiltrates/10 hepatic lobules. Hepatocellular necrosis was characterized by the presence of individual hepatocytes with an eosinophilic cytoplasm with or without fragmented or absent nuclei. (B) Liver/body weight ratio: The liver weights were evaluated as a ratio of overall body weight. Asterisks indicate statistical significance as determined by two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001.
Fig. 6

**A**

Mean frequencies of IFN-γ expressing CD4+ T cells among CD3+ cells

**B**

Mean frequencies of IL-4 expressing CD4+ T cells among CD3+ cells

**C**

Mean frequencies of CD3+CD4+ T cells

**Fig. 6.** Frequencies of IFN-γ and IL-4 intracellular cytokines in mononuclear cells isolated from spleen and mesenteric lymph node tissues and PBMCs from peripheral blood of wild-type and Jh knockout piglets experimentally-infected with HEV. PBMCs and MNCs were collected from each piglet at 28 days post-inoculation (dpi) at necropsy. Cells were gated on CD3 and stained for extracellular and intracellular markers CD4+ and IFN-γ and IL-4, respectively, after stimulation with HEV-specific capsid protein. The mean frequencies of (A) IFN-γ, (B) IL-4, (C) CD4+, are indicated and expressed as mean +/- SEM. All frequencies were determined by flow cytometry with 100,000 events per sample. *p<0.05.
Fig. 7. Frequencies of TGF-β and IL-10 intracellular cytokines in mononuclear cells isolated from spleen and mesenteric lymph node tissues and PBMCs from peripheral blood of wild-type and Jh knockout piglets experimentally-infected with HEV. PBMCs and MNCs were collected from each piglet at 28 days post-inoculation (dpi) at necropsy. Cells were gated on CD3 and stained for extracellular and intracellular markers CD4+, CD25+, Foxp3, and TGF-β and IL-10 respectively after stimulation with HEV-specific capsid protein. The mean frequencies of (A) CD25+Foxp3+, (B) CD25−Foxp3+, (C) TGF-β, and (D) IL-10 are indicated and expressed as mean +/- SEM. All frequencies were determined by flow cytometry with 100,000 events per sample.
Chapter V: Evidence for an Unknown Agent Antigenically Related to the Hepatitis E Virus in Dairy Cows in the United States

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ABSTRACT

Genotypes 3 and 4 hepatitis E virus (HEV) strains within the species *Orthohepevirus A* in the family *Hepeviridae* are zoonotic. Recently, a genotype 4 HEV was reportedly detected in fecal samples of cows; although, independent confirmation is lacking. In this study, we first tested serum samples from 983 cows in different regions in the United States for the presence of IgG anti-HEV and found that 20.4% of cows were seropositive. The highest seroprevalence rate (68.4%) was from a herd in Georgia. In an attempt to genetically identify HEV from cattle, a prospective study was conducted in a known seropositive dairy herd by monitoring 10 newborn calves from birth to 6 months of age for evidence of HEV infection. At least three of the 10 calves seroconverted to IgG anti-HEV, and importantly, the antibodies present neutralized genotype 3 human HEV, thus indicating the specificity of IgG anti-HEV in cattle. However, our extensive attempts using broad-spectrum RT-PCR assays and MiSeq deep-sequencing technology to genetically identify HEV-related sequences in cattle failed. The results suggest the existence of an agent antigenically-related to HEV in cattle; although, contrary to published reports, we showed that the IgG recognizing HEV in cattle was not caused by HEV infection.

**Key words**: Hepatitis E virus (HEV); Cattle; Bovine; Zoonotic transmission; Neutralizing antibodies; Seroprevalence
1. INTRODUCTION

Hepatitis E virus (HEV), the causative agent of hepatitis E, is transmitted via fecal-oral route through contaminated water and food.\textsuperscript{1} Hepatitis E is a global disease with large waterborne outbreaks occurring in developing countries due to poor sanitation conditions. In industrialized countries, sporadic and cluster cases of hepatitis E due to the ingestion of contaminated animal meats and shellfish or direct contact with infected animals have also been reported.\textsuperscript{2} The overall mortality rate associated with HEV infection ranges from 0.5%-4% in immunocompetent individuals; however, concurrent pregnancy accounts for increases in mortality up to 28%.\textsuperscript{3} \textsuperscript{4} Immunocompromised individuals infected with HEV, such as organ transplant recipients,\textsuperscript{5} lymphoma and leukemia patients,\textsuperscript{6} \textsuperscript{7} or patients with HIV infection,\textsuperscript{8} tend to progress into chronicity.\textsuperscript{9}

HEV is a recognized zoonotic agent with numerous known animal reservoirs.\textsuperscript{10} Among the well-characterized animal strains of HEV are the genotypes 3 and 4 swine HEV within species \textit{Orthohepevirus A} in domestic and wild pigs,\textsuperscript{11} \textsuperscript{12} and the genotypes 1-4 avian HEV within species \textit{Orthohepevirus C} in chickens.\textsuperscript{13} \textsuperscript{14} The recent identification of genetically-diversified strains of HEV across a wide range of animal species including bat,\textsuperscript{15} fish,\textsuperscript{16} rat,\textsuperscript{17} ferret,\textsuperscript{18} rabbit,\textsuperscript{19} wild boar,\textsuperscript{12} moose,\textsuperscript{20} mongoose,\textsuperscript{21} deer,\textsuperscript{22} and camel\textsuperscript{23} greatly expanded the host range and diversity of the virus. Ruminant species including deer,\textsuperscript{22} goats,\textsuperscript{24} sheep, and cattle\textsuperscript{25} have been implicated as potential reservoir as either IgG anti-HEV or viral RNA have been detected in these species. In 2016, genotype 4 HEV RNA was reportedly detected in cows in China, and raw milk was shown to be contaminated with infectious HEV.\textsuperscript{26} Surprisingly, the HEV sequence from cows were more than 99% identical across the entire genome to the HEV sequences from humans and pigs in China.\textsuperscript{26} Unfortunately, independent confirmation of this finding is lacking. Likewise, Yan et al.
reported 8 of 254 yellow cattle in Shandong, China with detectable genotype 4 strain HEV RNA from serum.\textsuperscript{27} However, two more recent and independent studies failed to identify HEV RNA in milk and fecal samples from >10\% of cows in Belgium\textsuperscript{28} or from bulk milk samples from dairy herds in Germany\textsuperscript{29}. Therefore, the main objective of this study was to determine if cattle in the United States are infected with HEV.

2. MATERIALS AND METHODS

2.1. Bovine serum samples

A total of 1,168 serum samples were collected from 983 cows in different regions of the United States including 223 serum samples from a university dairy herd in Virginia, 732 archived serum samples from feed lot cattle herds located in Texas, Oklahoma, New Mexico, South Dakota, North Dakota, Montana, Wyoming, Iowa, and Nebraska, and a longitudinal study including 213 serum samples from 38 cows in a cattle herd in Georgia collected at different timepoints (Table 1).

2.2. A prospective field study to genetically identify HEV from calves

In an attempt to genetically identify HEV from cows, we conducted a prospective study in a known HEV IgG seropositive university dairy herd in Virginia by following 10 newborn calves from birth to 6 months of age when they were moved to pasture. Weekly blood and fecal samples were collected from each of the 10 calves. Newborn calves were housed in individual hutches until 12 weeks of age, when they were moved to group barn housing under the standard operating procedures at this farm. The weekly serum and fecal samples were tested for the presence of HEV RNA by a nested universal RT-PCR assay that is capable of detecting genotypes 1-4 HEV strains (Table S1) and the weekly serum samples were also tested by a commercial ELISA kit (Wantai,
China) for the presence of IgG anti-HEV (Fig. 1). Additionally, the serum level of liver enzymes, including gamma-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), and aspartate aminotransferase (AST), were also measured (Fig. 2).

2.3. Collection of environmental samples from an HEV IgG positive dairy farm

A total of 100 environmental samples were collected from an HEV IgG seropositive dairy farm in Virginia and stored at -80°C until testing. These included composite fresh fecal samples from the milking parlor floor, pastures, floors and bedding from cow and calf barns, run-off water and fecal samples from the waste collection tanks of the associated barns, and standing pools of cleaning water. All environmental samples were tested for the presence of HEV RNA by a broad-spectrum nested RT-PCR assay.

2.4. Enzyme-linked immunosorbent assay (ELISA) to detect IgG anti-HEV in bovine sera

The bovine serum samples were tested for IgG anti-HEV using the commercial Wantai Double Antigen Sandwich ELISA kit (Beijing Wantai Biological Pharmacy Enterprise CO; https://www.szabo-scandic.com/fileadmin/content/images/AAH/HEV/HEV-IgG_CE_IFU_1_.pdf). This commercial assay has been shown to be superior to other assays in detecting HEV antibodies in a previous study.30 10μL of each serum sample was diluted into 100μL of provided diluent, tested in duplicate wells coated with recombinant HEV antigens to ORF2 (aa 394-606), and HEV antibodies bound using horse-radish peroxidase (HRP-) conjugated rabbit anti-human IgG secondary antibody, according to the manufacturer’s instructions. Substrate conversion absorbance was read using a Tecan Sapphire 2.0 instrument using Magellan software. Specific signal was collected using 405 nm with a light scatter reference wavelength of 630 nm.
The results for each serum sample were calculated by comparing each individual sample’s optical density (S) to the cut-off value (C.O.) following the manufacturer’s instructions and quality control criteria. Serum samples with S/C.O.>1.0 were considered positive for IgG binding HEV. Results were declared borderline when S/C.O. between 0.9 and 1.1 was measured, and these samples were re-tested for confirmation. Samples with S/C.O.<1.0. were declared negative for IgG binding HEV. All sera from herds from different states (Table 1), the Virginia dairy milking herd (Table 1), and the intensively studied calves and their respective dams (Table 2) were tested for the presence of IgG binding HEV by ELISA (Fig. 1).

2.5. Serum level of liver enzyme analyses

All sera from the calves in the prospective study (ID #’s 5078, 5079, 5080, 5081, 5082, 5083, 5084, 5086, 5088, and 5091) were tested by the Cornell Animal Health Diagnostic Center (Ithaca, NY) using standard methods for serum levels of liver enzymes including gamma-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), and aspartate aminotransferase (AST). Reference intervals of each enzyme specifically for bovine as determined by Cornell AHDC are as follows: AST 61-162 U/L, GLDH 11-83 u/L, and GGT 9-50 U/L (Fig. 2).

2.6. Broad-spectrum nested RT-PCR assays to detect HEV RNA in bovine samples

Selected serum, fecal, and environmental samples were tested for the presence of HEV using a broad-spectrum RT-PCR assay that is known to detect RNA of multiple HEV genotypes as previously described. Additionally, degenerate primer sets were also designed based on the alignment of known HEV strains (Table S1) and used for the detection of HEV RNA in the same nested RT-PCR assay. Furthermore, published primers used successfully to identify novel animal
strains of HEV\textsuperscript{20, 32} were similarly used in the same RT-PCR assay. RNA was extracted from approximately 100 µL of the 10% fecal suspension or 100 µL of serum samples using TriReagent (Molecular Research Center, Cincinnati, OH, USA). cDNA was prepared using Superscript II reverse transcriptase (Life Technologies; Thermo Fisher, Waltham, MA). Multiple primer sets were utilized for both the first and second rounds of nested RT-PCR (Table S1) using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA).

For the serum samples from calves in the prospective study, an additional hemi-nested touchdown RT-PCR was utilized with degenerate primers BatHEVF4228 and BatHEVR4598 (Table S1) according to the published protocol.\textsuperscript{15} The second round of the nested RT-PCR was completed using 5µL of first round DNA product as the template and degenerate primers BatHEVF4228 and Bat HEVR4565 (Table S1).\textsuperscript{15}

2.7. \textit{In vitro} neutralization assay for HEV

A subclone of the Huh7 human liver cell line, Huh7-S10-3,\textsuperscript{33} was utilized to propagate the genotype 3 human HEV Kernow P6 strain.\textsuperscript{34} Human hepatocellular carcinoma cells HepG2/C3A (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; ThermoFisher Scientific, Ontario, Canada) with 10% FBS on collagen coated flasks and used for all \textit{in vitro} infectivity assays as described previously\textsuperscript{35, 36} on selected bovine sera that were tested positive by ELISA. Briefly, the genotype 3 HEV Kernow P6 virus stock \textsuperscript{37} was incubated in duplicate with PBS as the negative control, Chimp 1313 hyperimmune anti-HEV serum as the positive control, or selected bovine serum samples at 1:10, 1:100, and 1:1000 dilutions. The mixture was then added onto the HepG2/C3A liver cells and incubated for 2 hr at 37°C. Afterwards, the samples were removed, cells washed with PBS, and fresh DMEM medium was
added and incubated for 5-6 days. Cells were stained at 5-6 days post-infection by immunofluorescence assay (IFA) with a rabbit antisera against a bacterially-derived 6x His capsid protein containing a 111 N-terminal amino acid truncation from the HEV Kernow-C1-P6 strain\textsuperscript{34; 38} as the primary antibody and AlexaFluor 488 goat anti-rabbit IgG (H&L) (Molecular Probes, Life Technologies, Carlsbad, CA, USA) as the secondary antibody. The number of positive cells were counted and recorded as the number of fluorescein focus units (FFU). Serum samples were compared to the negative control (PBS) to generate a value for overall percent decrease in HEV infectivity, which represented the ability of the bovine serum to neutralize genotype 3 human HEV. Sera were scored as effective if there was greater than 50% reduction in infectivity and further titered at 1:2 to 1:8192 serial dilutions using the same procedure (Table 3).

2.8. Deep sequencing of serum and fecal samples attempting to genetically identify HEV sequences from bovine samples

Selected serum samples from calves in the prospective study were deep-sequenced using MiSeq at the Biocomplexity Institute of Virginia Tech, Blacksburg VA, and the Blood Systems Research Institute at the University of California, San Francisco, CA. Serum samples from calf numbers 5082 and 5091 were selected for deep-sequencing by MiSeq techniques over the time course of collection. Additionally, serum samples from all calves (5078, 5079, 5080, 5081, 5082, 5083, 5084, 5086, 5088, and 5091) were pooled and deep-sequenced via MiSeq for the presence of HEV-related sequences across the trial as well. Furthermore, 100 serum samples representing all of the different geographic regions collected were randomly selected, and 5 µL from each serum was used to assemble a single pool that was subsequently deep-sequenced. Selected fecal samples from calves in the prospective study including calves #s 5082 and 509, were deep-sequenced using
MiSeq at Columbia University, New York, NY. Library preparation and deep-sequencing analysis was performed as described previously.39-41

3. RESULTS

3.1. Prevalence of IgG binding HEV antigen in bovine herds in the United States

Serum samples from a total of 983 individual cows including feedlot cows from 9 different States, a dairy herd in Virginia, and a herd in Georgia were tested for IgG that bound HEV antigen using a commercial ELISA assay. The overall seroprevalence rate across all herds was 20.4% with a range of 16.1% to 68.4% as associated with the geographic locations of the herds (Table 1). The herd from Georgia was unique in that sequential serum samples were collected from each of the 38 cows over the course of 9 months. The seroprevalence rate across all 213 serum samples from the 38 cows within the Georgia herd was 24.4%. However, this corresponded to 68.4% of individual cows being seropositive at least once during the study. Of the 223 individual cows from the Virginia Dairy herd, 10 serum samples were from the dams of the calves participating in the prospective study. Dam numbers 4682, 4810, and 4700, the mothers of calf numbers 5086, 5091, and 5088, were positive for IgG anti-HEV within 2 weeks of calving. In contrast, dam numbers 4832, 4855, 4517, 4854, 4852, 5686, and 4344 were not positive for IgG recognizing HEV (Table 2).

3.2. Seroconversion to IgG binding HEV antigen in serum of calves from a prospective study in a Virginia Dairy herd

For the prospective study in a dairy herd, the 10 female calves were born from both seropositive dams (#’s 4682, 4810, and 4700) and seronegative dams (#’s 4832, 4855, 4517, 4854, 4852, 4686,
Calf numbers 5079, 5080, 5081, 5084, and 5088 did not seroconvert to HEV at any time point during the study, and were considered negative (Fig. 1a). Calf numbers 5078, 5082 and 5091 had varying levels of seroconversion. Calf #5078 briefly seroconverted by producing IgG recognizing HEV at 31 days of age for 1 week, and again at the age of 71 days for 2 weeks with a relatively low level of antibody (Fig. 1b). Calf #5082 briefly seroconverted at 28 days of age for 1 week, and again at 78 days of age with a high level of IgG recognizing HEV observed and lasting 9 weeks. Calf #5091 seroconverted at 106 days of age, with a high level of HEV IgG observed for 3 weeks. The remaining two calves 5083 and 5086 both had only transient seroconversion with very low levels of antibodies (Fig. 1b).

The prospective study ended with the movement of calves to pasture at 6 months of age. None of the 10 calves were positive for maternal IgG anti-HEV at the time of birth or within the first 3 weeks after birth (Table 2). Dam numbers 4682, 4700, 4810, corresponding to calf numbers 5086, 5088, and 5091, had HEV IgG within 2 weeks prior to calving. No correlation between the seropositive dams and the seroconversion of calves was observed in this study (Table 2).

3.3. Elevation of serum level of glutamate dehydrogenase (GLDH) liver enzyme in calves that seroconverted to IgG anti-HEV

Since at least three calves in the prospective study seroconverted to HEV, we measured the serum levels of the liver enzymes; AST, GLDH, and GGT in all serum samples from each of the ten calves on a bi-monthly basis in (Fig. 2). The results showed that the AST values remained within normal limits (62-162U/L) for all calves throughout the study. The GGT values were initially elevated on the day of birth, and during the first two weeks of life in calf numbers 5088 and 5091. This is expected in neonates. However, all subsequent samples remained within normal limits for
all calves (11-83 u/L). There appears to be a correlation between peaks in serum levels of GLDH and the levels of IgG recognizing HEV among the calves that seroconverted in this prospective study (Fig. 3). Calf numbers 5078, 5082, and 5091, which had relatively high levels of IgG recognizing HEV, also had high serum levels of GLDH at the same time points (Fig. 3).

3.4. Failure to detect HEV-related sequences in calves from the prospective study by broad-spectrum nested RT-PCR assays

In an attempt to detect HEV RNA from calves in the prospective study, we utilized broad-spectrum nested RT-PCR assays with different sets of primers to test selected fecal and serum samples from all calves. PCR primers targeting the sequences from known HEV strains in the conserved regions of ORF1 (RNA-dependent RNA polymerase region), ORF2 (capsid gene region), and ORF3 were used for the RT-PCR assays. Furthermore, published RT-PCR primers and protocols utilized for recent detection of bat,\textsuperscript{15} rat,\textsuperscript{31} and rabbit HEV\textsuperscript{32} strains, and degenerate pan-primers used for detection of multiple HEV genotypes were also used in our repeated attempts to amplify HEV RNA (Table S1). Serum and fecal samples used for these RT-PCR assays included three calves (ID no. 5078, 5082, and 5091) that had seroconverted to HEV (Fig. 1b). Additional samples collected from many time points from the remaining calves were assayed as well. Despite repeated testing with different sets of primers and protocols, none of the bovine serum or fecal samples in the prospective study were positive for HEV RNA. A serum sample containing rabbit HEV was used as the positive control and was positive in all RT-PCR reactions. Similarly, environmental samples from the dairy herd including water run-off, surface fecal samples, and combined samples from the sick cow area, calf hutches, calving pen, and free-stall barns all tested negative using these RT-PCR assays.
3.5. MiSeq deep-sequencing of various bovine serum and fecal samples did not reveal the presence of HEV-related sequence

The failure to detect HEV RNA by broad-spectrum RT-PCR assays from serum and fecal samples of calves that apparently seroconverted to HEV prompted us to employ a deep-sequencing strategy. First, we deep-sequenced pooled sera from the calves that seroconverted to HEV including samples collected at the week of seroconversion, three time points prior to seroconversion, and the week following seroconversion. However, analysis of the deep sequence reads did not identify any HEV-related sequences from the samples. Therefore, we performed additional MiSeq deep sequencing on combined serum samples collected from calves 5082 and 5091 at all time points from the date of birth to the end of the prospective study. Again, HEV-related sequences were not identified. Additionally, we performed MiSeq deep sequencing on fecal samples from calves 5082 and 5091 at all time points, but did not identify any HEV-related sequence. Sequences from numerous other viruses were identified in the serum and fecal samples of both calves (data not shown), indicating that the MiSeq deep-sequencing technique was successfully applied. Furthermore, MiSeq deep sequencing was performed on a pooled serum sample from all time points of all ten calves in the prospective study, again with HEV-related sequence was not detected. Finally, we performed MiSeq deep sequencing on a combined serum sample from a total of 100 cattle randomly selected from 9 different states, and again HEV-related sequence was not identified.

3.6. IgG antibody recognizing HEV antigen present in calves are capable of neutralizing genotype 3 human HEV
A virus neutralization assay was performed to further validate the ELISA results. We found that serum samples with individual ELISA S/C.O. values ranging from 0.145-0.957 (1.0 is considered seropositive) were unable to neutralize genotype 3 human HEV in HepG2/C3 cells (data not shown). However, three selected serum samples from the Virginia dairy herd with individual S/C.O. values of 2.270, 20.851, and 2.658 (Table 3), respectively, consistently reduced the infection of genotype 3 human HEV by >50% (Table 3). Reductions of ~50% corresponded with virus neutralization titers of 1:16 for calves no. 5078 and 5082 (S/C.O. values 2.658 and 2.270, respectively).

4. DISCUSSION

HEV has been genetically identified from more than a dozen animal species. Genotypes 3 and 4 HEV strains within the species Orthohepevirus A infect across species barriers and are zoonotic. Swine represent the primary natural reservoir for genotypes 3 and 4 HEV, and chronic and neurologic cases of hepatitis E in humans are almost exclusively caused by the zoonotic genotype 3 HEV of likely animal origins. Since the discovery of swine HEV from pigs in the United States in 1997, genotypes 3 and 4 HEV have been detected worldwide from both wild and domestic swine. The ubiquitous nature of swine HEV in pigs worldwide has led to contamination of pork products including the United States, the Netherlands, the United Kingdom, and Japan. Food-borne transmission of swine HEV has been definitively linked to the consumption of raw or undercooked pork products.

The known host range of HEV is rapidly expanding with the genetic identification of novel HEV strains from a wide range of species. HEV IgG has also been identified in several other species including cats, dogs, cattle, horses, and goats, although the sources of virus inducing
seroconversion in these species are unclear. Recently, a genotype 4 HEV was reportedly detected from fecal samples of cows, and surprisingly the infected cows reportedly excreted virus into milk.\textsuperscript{26} This report, although still lacking independent confirmation, raises serious concerns regarding milk and beef safety. On the contrary, reports from Germany\textsuperscript{29} and Belgium\textsuperscript{28} failed to identify the presence of HEV in milk samples and dairies. Therefore, it is imperative to definitely determine the nature and extent of HEV infection in cattle.

In this study, first we utilized a commercial ELISA assay to determine the seroprevalence of IgG recognizing HEV antigen in cattle in the United States. Serum samples from a total of 983 individual cows from 9 different states were tested, and the results showed that the overall seroprevalence rate was 20.4\% with herd seroprevalence rates ranging from 16.1\% to 68.4\% depending upon the geographic locations. The seroprevalence in cattle was similar to those reported in other animal species.\textsuperscript{43; 48} We also tested 213 sequential serum samples collected from 38 cows in Georgia over a period of 9 months, and found that 68.4\% of the cows were seropositive at least once during that period, which is similar to the levels observed in some swine herds.\textsuperscript{49; 50} The results suggest that there is an HEV-related agent in the bovine population.

In our attempt to identify HEV from cattle, we conducted a prospective study in a known HEV-seropositive dairy herd by monitoring 10 newborn calves from the time of birth to 6 months of age for evidence of HEV infection. We showed that at least three of the 10 calves temporarily seroconverted to IgG against HEV at different time points during the course of the prospective study, suggesting that a HEV-related agent is present in these calves. Importantly, we demonstrated that the IgG anti-HEV present in the seropositive calves show some neutralizing activity against the genotype 3 human HEV. This indicates that HEV IgG present in the cattle can target antigenic epitopes found on HEV. Interestingly, the serum level of liver enzyme GLDH
appears to correlate with the seroconversion to HEV IgG in the calves. This is suggestive of potential liver damage associated with infection by the HEV-related agent.

Our repeated and extensive attempts to genetically identify the source of seropositivity in the cattle were unsuccessful. Both HEV-specific and broad-spectrum degenerate primers that are known to amplify sequences from multiple HEV genotypes with the species Orthohepevirus A as well as published RT-PCR primers that were successfully used to identify other novel animal strains of HEV from moose, rabbit, and bat all failed to amplify HEV RNA from serum or fecal samples collected from the three calves that had seroconverted to HEV and the seven seronegative calves. Similarly, broad-spectrum RT-PCR assays also failed to amplify HEV RNA from the environmental samples collected in the seropositive Virginia dairy herd.

Detection of viral RNA from ruminant species, especially from fecal samples, present hurdles when utilizing RT-PCR due to the presence of potential inhibitors. While serum samples, which typically do not have PCR inhibitors, were also used for RT-PCR detection in the study, viremia is transient during HEV infection and thus it is possible that the window of HEV RNA detection may have been missed. It is also possible that the HEV responsible for the seropositivity in cattle is genetically highly divergent, and therefore these broad-spectrum RT-PCR assays still may not be able to detect the virus. For example, the avian HEV in chickens shares only approximately 50% nucleotide sequence identity with mammalian HEVs. The HEV from cutthroat trout shares <27% identity with mammalian HEVs, and the moose HEV shares only approximately 37-63% identity with other known HEV strains. Therefore, deep sequencing of the bovine samples was also performed to identify all viral sequences present in cattle.

MiSeq deep sequencing was performed on serum samples from the calves that had seroconverted to HEV in the prospective study including calf numbers 5082 and 5091 at all time
points, fecal samples from calves 5082 and 5091, and on pooled serum samples from 100 cows in different geographic regions of the United States. Despite repeated attempts, no HEV-related sequence was identified from the deep sequencing reads, even though specific sequences from other bovine viruses were successfully identified in the samples, indicating that the MiSeq deep sequencing procedures worked well.

In conclusion, we demonstrate serological evidence for the existence of an HEV-related agent in cattle, as documented by the relatively high seroprevalence rate of IgG recognizing HEV in cattle. Further, seroconversion to HEV in newborn calves born seronegative over the course of their calf-hood, and the ability of that antibody to neutralize genotype 3 human HEV in cell culture strongly indicate an antigenic relationship with an agent related to HEV. The failure of our repeated attempts to genetically identify HEV from cows and calves using broad-spectrum RT-PCR assays and MiSeq deep sequencing technology suggests an important limitation in the interpretation of HEV serological data reported in a large number of animal species including cattle. It is possible that the seroconversion in cattle is caused by antigenic cross-reaction with a related, but as yet unknown agent or alternatively is due to antigenic cross-reactivity with unknown host protein(s). Unless HEV can be definitively and reproducibly identified from cattle, we must be very cautious in speculating the role of cattle, if any, in HEV transmission and potential zoonotic disease.

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human HEV genotype 3 Kernow P6 strain, Huh7-S10-3 cells, and anti-HEV hyperimmune chimpanzee 1313 antiserum. We also thank Dr. Amit Kapoor of Columbia University, NY, and Dr. Saikumar Karyala and Dr. Bob Settlage of Biocomplexity Institute of Virginia Tech for their expert assistance in MiSeq deep sequencing of the bovine samples.

**Conflicts of interest:** The authors declare that they have no competing interests.

**Disclaimers:** This paper has not been published elsewhere in part or in entirety, and is not under consideration for publication by another journal.
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Table 1. Prevalence of IgG binding to HEV in cattle from different geographic regions of the United States

<table>
<thead>
<tr>
<th>Herd Locationa</th>
<th>Year samples collected</th>
<th>No of cows sampled</th>
<th>Percent (%) positive for IgG anti-HEV</th>
<th>Range of Individual S/C.O. valuesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX, OK, NM</td>
<td>2010</td>
<td>352</td>
<td>17.84</td>
<td>0.16-21.5</td>
</tr>
<tr>
<td>SD, ND, MT, WY</td>
<td>2010</td>
<td>250</td>
<td>19.60</td>
<td>0.15-26.5</td>
</tr>
<tr>
<td>SD, IA, NE</td>
<td>2010</td>
<td>130</td>
<td>22.30</td>
<td>0.15-14.4</td>
</tr>
<tr>
<td>GA</td>
<td>2009</td>
<td>38</td>
<td>68.4</td>
<td>0.18-24.8</td>
</tr>
<tr>
<td>VA Dairy</td>
<td>2012</td>
<td>223</td>
<td>16.1</td>
<td>0.07-7.56</td>
</tr>
</tbody>
</table>

*aSamples listed by geographic locations as follows: Texas (TX), Oklahoma (OK), New Mexico (NM), South Dakota (SD), North Dakota (ND), Montana (MT), Wyoming (WY), Iowa (IA), Nebraska (NE), Georgia (GA), and Virginia (VA).

*bAll sera were tested by an established commercial ELISA assay (Wantai ELISA kit). Individual ELISA S/C.O. value >1.0 is considered positive.
Table 2. Serological evidence of HEV IgG in dams and their corresponding calf from the prospective study in a university dairy herd

<table>
<thead>
<tr>
<th>Dam ID</th>
<th>Date of sample collection</th>
<th>Dam S/C.O. anti-HEV value</th>
<th>Calf ID</th>
<th>Calf S/C.O. anti-HEV value at birth</th>
<th>Seroconversion of calf to IgG anti-HEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>4832</td>
<td>11/7/12</td>
<td>0.4879</td>
<td>5078</td>
<td>0.2070</td>
<td>Yes</td>
</tr>
<tr>
<td>4855</td>
<td>11/7/12</td>
<td>0.3532</td>
<td>5082</td>
<td>0.1925</td>
<td>Yes</td>
</tr>
<tr>
<td>4517</td>
<td>11/7/12</td>
<td>0.263</td>
<td>5083</td>
<td>0.1590</td>
<td>Transient</td>
</tr>
<tr>
<td>4682&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11/7/12</td>
<td>5.668&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5086</td>
<td>0.5790</td>
<td>Transient</td>
</tr>
<tr>
<td>4810&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11/7/12</td>
<td>2.5926&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5091</td>
<td>0.2248</td>
<td>Yes</td>
</tr>
<tr>
<td>4854</td>
<td>11/7/12</td>
<td>0.3444</td>
<td>5079</td>
<td>0.1850</td>
<td>No</td>
</tr>
<tr>
<td>4852</td>
<td>11/7/12</td>
<td>0.693</td>
<td>5080</td>
<td>0.2217</td>
<td>No</td>
</tr>
<tr>
<td>4686</td>
<td>11/7/12</td>
<td>0.4826</td>
<td>5081</td>
<td>0.1632</td>
<td>No</td>
</tr>
<tr>
<td>4344</td>
<td>11/7/12</td>
<td>0.5624</td>
<td>5084</td>
<td>0.2008</td>
<td>No</td>
</tr>
<tr>
<td>4700&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11/7/12</td>
<td>7.5513&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5088</td>
<td>0.3733</td>
<td>No</td>
</tr>
</tbody>
</table>

<sup>a</sup> Dams and their individual S/C.O. values considered positive for IgG recognizing HEV.
<sup>b</sup> Each of the calves was born between November 4<sup>th</sup> and November 25<sup>th</sup>, 2012 with the Dams sampled within 2 weeks of the expected calving dates.
<sup>c</sup> IgG anti-HEV ELISA O.D. (optical density) values reported as a ratio of the sample to the average of three negative controls plus 0.12 as instructed by the commercial Wantai ELISA assay. Individual ELISA S/C.O. >1.0 is considered positive.
<sup>d</sup> Calves seroconverted to HEV by producing IgG during the course of the prospective study. Two calves (no. 5083, and 5086) only transiently seroconverted for only 1 or 2 weeks.
Table 3. Neutralizing capability of selected bovine serum samples from calves on the infectivity of a genotype 3 human HEV (Kernow P6 strain) in HepG2/C3A human liver cells

<table>
<thead>
<tr>
<th>Bovine Serum ID and controls</th>
<th>Date of sample collection</th>
<th>ELISA O.D. value</th>
<th>Individual S/C.O. value</th>
<th>% Decrease in HEV infectivity(^a)</th>
<th>Sample neutralizing antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>Chimp 1313</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>91.87</td>
<td>N/A</td>
</tr>
<tr>
<td>5078</td>
<td>1/14/13</td>
<td>0.411</td>
<td>2.658</td>
<td>48.56</td>
<td>1:16</td>
</tr>
<tr>
<td>5082</td>
<td>12/5/12</td>
<td>0.340</td>
<td>2.270</td>
<td>46.9</td>
<td>1:4</td>
</tr>
<tr>
<td>5082</td>
<td>1/10/13</td>
<td>3.125</td>
<td>20.851</td>
<td>49.28</td>
<td>1:16</td>
</tr>
</tbody>
</table>

\(^a\)All bovine sera were tested in dilutions 1:10, 1:100 and 1:1000, and were also titered in serial dilutions from 1:2 to 1:8192 in a serum virus neutralization assay. Chimp 1313 is a hyperimmune HEV antisera as a positive control with at least 10^5 IgG ELISA titer against HEV from a chimpanzee experimentally-infected with HEV.
Supplementary Table 1. Oligo primers used for the detection of HEV RNA in fecal and serum samples by broad-spectrum nested RT-PCR assays

<table>
<thead>
<tr>
<th>Primer ID&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence</th>
<th>Tm</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan F1</td>
<td>5’-TCGCGCATACMTTYTTCCARA-3’</td>
<td>58.6</td>
<td>470</td>
</tr>
<tr>
<td>Pan R1</td>
<td>5’-GCCATGTTTCCAGACDGTRTTCCA-3’</td>
<td>59.0</td>
<td>470</td>
</tr>
<tr>
<td>Pan F2</td>
<td>5’-TGTGCTCTGTTGCGCCNTGTTYMG-3’</td>
<td>62.7</td>
<td>330</td>
</tr>
<tr>
<td>Pan R2</td>
<td>5’-CCAGGGCTACCRGARTGYTTCTTCCA-3’</td>
<td>63.4</td>
<td>330</td>
</tr>
<tr>
<td>USRAB DEG F1</td>
<td>5’-GCMACACGKTYYATGAARGA-3’</td>
<td>53.4</td>
<td>200</td>
</tr>
<tr>
<td>USRAB DEG R1</td>
<td>5’-ACYTTRGACCACATCVAGRGARC-3’</td>
<td>55.8</td>
<td>200</td>
</tr>
<tr>
<td>USRAB DEG F2</td>
<td>5’-GCTGAYACRCCRTCTTYGGY-3’</td>
<td>56.2</td>
<td>200</td>
</tr>
<tr>
<td>USRAB DEG R2</td>
<td>5’-TGAMGGRGTRGTYGRTCYT-3’</td>
<td>58.7</td>
<td>200</td>
</tr>
<tr>
<td>ORF2 BOVF1</td>
<td>5’-GGBCTNCCGACAGAATTRAT-3’</td>
<td>54.0</td>
<td>406</td>
</tr>
<tr>
<td>ORF2 BOVR1</td>
<td>5’-TRCCHGCTTCCAGRAGGA-3’</td>
<td>59.7</td>
<td>406</td>
</tr>
<tr>
<td>ORF2 BOVF2</td>
<td>5’-CYGTYGTSTCRGGCAATGG-3’</td>
<td>57.7</td>
<td>225</td>
</tr>
<tr>
<td>ORF2 BOVR2</td>
<td>5’-GWGWAASECSCAMARMACATCA-3’</td>
<td>52.4</td>
<td>225</td>
</tr>
<tr>
<td>ESP MOOSE F</td>
<td>5’-CATGGAAGGTGGTCAGGTT-3’</td>
<td>56.4</td>
<td>383</td>
</tr>
<tr>
<td>EAP MOOSE R</td>
<td>5’-AGGTTGCGGGGTCCGGGA-3’</td>
<td>71.7</td>
<td>383</td>
</tr>
<tr>
<td>BATHEVF4228</td>
<td>5’-ACYYTTYTGTGCYTTYTTGGTCCITGTT-3’</td>
<td>63.8</td>
<td>371/338</td>
</tr>
<tr>
<td>BATHEVR4598</td>
<td>5’-GCCATGTTCAGAGYGGTCTTCCA-3’</td>
<td>60.8</td>
<td>371</td>
</tr>
<tr>
<td>BATHEVR4565</td>
<td>5’-CCGGGTTCRCCIGAGTGTTTCTTCCA-3’</td>
<td>65.2</td>
<td>338</td>
</tr>
</tbody>
</table>

<sup>a</sup>F1, F2, R1, and R2 indicated first round forward, second round forward, first round reverse, second round reverse, respectively.
Fig. 1. Seroconversion to IgG anti-HEV in calves in a closed university dairy herd in Virginia in a prospective study. Ten newborn calves born to both IgG anti-HEV seropositive and seronegative dams were monitored for evidence of HEV infection from the day of birth to 6 months of age. The weekly serum samples from all calves were tested by the commercial Wantai ELISA assay for IgG anti-HEV. (A): Calves tested negative for IgG anti-HEV in the prospective study; (B): Calves tested positive for IgG anti-HEV in the prospective study. The specific signal (individual ELISA O.D.) values were collected and plotted as S/C.O. (Y-axis) as a function of the ages of the animals (days after birth) indicating when the samples were collected. The ELISA cut-off value indicating sero positivity to IgG anti-HEV is indicated as a dotted line at 1.0 based on the commercial Wantai ELISA assay standard.
Fig. 2. Serum levels of liver enzymes over time in the prospective study in calves from the time of birth to 6 months of age. Serum samples were collected weekly from each calf beginning with the day of birth and continuing prospectively until approximately 6 months of age. Serum levels of liver enzymes including (A) aspartate aminotransferase (AST), (B) glutamate dehydrogenase (GLDH), and (C) gamma-glutamyl transferase (GGT) were measured in each serum sample at the Cornell University Animal Health Diagnostic Center. Normal limits of each enzyme were provided with the analysis based on the diagnostic laboratory’s standards for each specific test, which are indicated on each scatterplot graph as a dotted line. Each sample indicates an individual calf serum sample for each time point and are expressed as the mean +/- SEM. Analysis was completed using two-way ANOVA.
Fig. 3. Correlation between seroconversion and serum levels of GLDH in serum samples collected prospectively from calves from the time of birth to 6 months of age. Calves (A) #5078, (B) 5082, (C) 5083, (D) 5086, and (E) 5091 seroconversion to IgG anti-HEV correlated with the elevations in serum levels of GLDH. The specific signal (individual ELISA O.D.) values were plotted as S/C.O. (left y-axis) as a function of the age of the animal (days after birth). The ELISA cut-off value indicating seropositivity to IgG anti-HEV is indicated as a dotted line at 1.0 based on the commercial Wantai ELISA assay standard. The serum levels of GLDH were plotted (right y-axis) as a function of the age of the animal (days after birth). The upper normal limit was provided with the analysis based on the diagnostic laboratory’s standard for GLDH in bovine species and indicated as the dotted line at 83 u/L.
Chapter VI

GENERAL DISCUSSION

As discussed throughout the dissertation research, many underlying mechanisms of HEV infection including pathogenesis of disease, immune dynamics during the acute stage, transmission and reservoir species, and the host factors and virus determinants leading to progressive disease, are not well understood. The zoonotic risk of HEV is well established (1-3); however, the ever-expanding host range and identification of new animal reservoir species pose a significant public health concern. Additionally, current animal models for HEV research do not adequately replicate the course of infection seen in infections in humans (4), nor do they develop the level of pathogenicity and progression of disease seen in immunocompromised (5, 6) and pregnant populations (7).

The first objective of the dissertation research was to establish an immunoglobulin (Ig) heavy chain JH (−/−) knockout gnotobiotic pigs using CRISPR/Cas9 technology. This model aimed to deplete the B-lymphocyte population resulting in an inability to elicit a humoral response. The resultant gnotobiotic piglets with challenged with a genotype 3 human strain of HEV (US-2). The dynamics of acute HEV infection were then systematically determined in this unique and novel gnotobiotic pig model in an attempt to better mimic the course of acute HEV infections observed in humans. As the natural host for genotypes 3 and 4 (2, 8, 9) HEV infections in humans, the outbred conventional piglet model has long been used in HEV research to study HEV biology and cross-species infection (2, 10, 11). However, infection with HEV in conventional pigs is clinically asymptomatic with only mild to moderate hepatic lesions (12, 13). Compared to the wild-type
gnotobiotic piglets, the frequencies of B-lymphocytes in Ig heavy chain JH (-/-) knockout pigs were significantly lower despite retaining normal levels of other innate and adaptive T-lymphocyte cell populations. After infection with HEV, the wild-type gnotobiotic piglets had higher viral RNA loads in feces and sera when compared with the JH (-/-) knockout piglets, which suggests that the Ig heavy chain JH (-/-) knockout pigs decreased the level of HEV replication within the host. Both the HEV-infected wild-type and JH (-/-) knockout gnotobiotic piglets induced more pronounced lymphoplasmacytic hepatitis and hepatocellular necrosis lesions in the liver with discernible difference when compared with a conventional pig model. The HEV-infected JH (-/-) knockout pigs had significantly enlarged livers both grossly and as a ratio of liver/body weight when compared with the PBS mock-infected groups. Additionally, the serum level of the liver enzyme GGT was significantly elevated in the HEV-infected JH (-/-) knockout pigs and may indicate liver damage, since the predominant source of GGT is the biliary epithelium. Therefore, the novel gnotobiotic piglet model is a valuable tool to aid in future studies into HEV pathogenicity, an aspect which has been difficult to reproduce in the available animal model systems, thus far.

HEV has been genetically identified from more than one dozen animal species (14). Swine serve as the primary animal reservoir for zoonotic genotypes 3 and 4 within the species Orthohepevirus A (15, 16) and are definitively linked to human infections through zoonotic and foodborne transmission of HEV (17, 18). Recently reported identification of genotype 4 HEV in fecal samples of cows and excretion of virus into cow milk (19, 20) raises concerns regarding milk and beef safety. Contradictory reports from Europe (21, 22) failed to identify evidence of HEV infection in dairy cows. Therefore, in the second project of the dissertation, the main objective was to determine if cattle in the United States are infected with a bovine strain of HEV. We demonstrated evidence of an HEV-related agent in cattle with a high overall level of
seroprevalence at 20.4% and herd seroprevalence rates ranging from 16.1-68.4% depending on geographic location. In a prospective animal study, three of the 10 calves seroconverted to IgG against HEV and this antibody was capable of neutralizing genotype 3 human HEV. Interestingly, the elevated serum levels of GLDH appeared to correlate with the peak of seroconversion, which is suggestive of potential liver damage. The failure to provide definitive evidence of virus in cattle using broad-spectrum RT-PCR assays and MiSeq deep sequencing technology suggests a limitation in the interpretation of HEV serological data in species such as cattle. Additionally, without definitive genetic identification of virus in cattle, we must be cautious in speculating the role of cattle, if any, in HEV transmission and potential zoonotic infection.

In summary, much knowledge related to HEV infection, pathogenesis, and progression of disease is currently lacking. A challenge in filling these knowledge gaps lies in the extensive genetic diversity of animal strains of HEV and the unresolved determination of all potential reservoirs capable of transmitting virus to the human population. Additionally, the lack of an adequate cell culture model for in vitro work and limited availability of commercial diagnostic assays for all strains of HEV makes understanding the course of infection difficult. As this dissertation research has presented, the identification of better animal models for research and the identification of all animal reservoirs are key in fully understanding HEV and its impacts on public health.

All projects included in this dissertation provided many unforeseen complications and identified numerous limitations in this field. While some portions of this dissertation work carried a negative result connotation, the projects provided groundwork for follow-up studies in the areas of novel strain identification and transmission, immune responses of HEV, and novel animal model development. Future directions of this work lead directly to the use of the gnotobiotic piglet model
in a prolonged experimental period study in order to ascertain an understanding of the full course of infection. This research will provide an understanding of the immune dynamics during the acute and chronic stages of infection. With the ability to develop classical liver lesions seen in humans, the gnotobiotic piglet will fill much-needed knowledge gaps in HEV research. Additionally, the lack of a bovine strain of HEV identified during the course of this work, points to the need to develop better diagnostic assays with consistent analysis for HEV. Prior conflicting studies from several countries around the world, lead the scientific community to consider bovine as a potential reservoir for HEV. However, this study definitively determines that cattle herds in the United States do not carry a bovine strain of HEV. Overall, the projects in this dissertation answer important questions for the scientific community and will be directly linked to HEV studies carried out in our laboratory.
REFERENCES
