

# **Cross-protection and Potential Animal Reservoir of the Hepatitis E Virus**

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## **ABSTRACT**

HEV is an important public health concern due largely to water-borne outbreak. Recent research confirms individual cases of zoonotic transmission due to human exposure to contaminated animal meats. At least four recognized and two putative genotypes of mammalian HEV have been reported: genotypes 1 and 2 are restricted to humans whereas genotypes 3 and 4 are zoonotic. In addition to humans, strains of HEV have been genetically identified from pigs, chickens, rats, mongoose, deer, rabbits and fish. The current experimental vaccines are all based on a single strain of HEV, even though multiple genotypes of HEV are co-circulating in some countries and thus an individual may be exposed to more than one genotype. Therefore, it is important to know if prior infection with a genotype 3 swine HEV will confer protective immunity against subsequent exposure to genotypes 3 and 4 human and swine HEV. In the first study, specific-pathogen-free pigs were divided into 4 groups of 6 each. Pigs in the three treatment groups were each inoculated with a genotype 3 swine HEV, and 12 weeks later, challenged with the same genotype 3 swine HEV, a genotype 3 human HEV, and a genotype 4 human HEV, respectively. Sera from all pigs were tested for HEV RNA and IgG anti-HEV, and fecal samples were also tested for HEV RNA each week. The pigs inoculated with swine HEV became infected as evidenced by fecal virus shedding and viremia, and the majority of pigs also developed IgG anti-HEV prior to challenge at 12

weeks post-inoculation. After challenge, viremia and fecal virus shedding of challenge viruses were not detected, suggesting that prior infection with a genotype 3 swine HEV prevented pigs from developing viremia and fecal virus shedding after challenge with homologous and heterologous genotypes 3 and 4 HEV, respectively.

Immunogenic epitopes are located within the open reading frame 2 (ORF 2) capsid protein and recombinant ORF 2 antigens are capable of preventing HEV infection in non-human primates and chickens. In the second study we expressed and purified N-truncated ORF 2 antigens based on swine, rat, and avian HEV strains. Thirty pigs were randomly divided into groups of 6 pigs each and initially vaccinated with 200µg swine ORF 2 antigen, rat ORF 2 antigen, avian ORF 2 antigen, or PBS buffer (positive and negative control groups) and booster with the same vaccine 2 weeks later. At 4 wks, after confirming seroconversion to IgG anti-HEV antibody with ELISA, all groups except the negative control were challenged with swine genotype 3 HEV (administered intravenously). The protective and cross-protective abilities of these antigens were determined following swine genotype 3 challenge by evaluating both serum and fecal samples for HEV RNA using nested RT-PCR and IgG anti-HEV using ELISA. The results from these two studies have important implications for future development of an effective HEV vaccine.

As a part of our ongoing efforts to search for potential animal reservoirs for HEV, we tested goats from Virginia for evidence of HEV infection and showed that 16% (13/80) of goat sera from Virginia herds were positive for IgG anti-HEV. Importantly, we demonstrated that selected goat sera were capable of neutralizing HEV in cell culture. Subsequently, in an attempt to genetically identify the HEV-related agent from goats, we

conducted a prospective study in a closed goat herd with known anti-HEV seropositivity and monitored a total of 11 kids from the time of birth until 14 weeks of age for evidence of HEV infection. Seroconversion to IgG anti-HEV was detected in 7 out of the 11 kids, although repeated attempts to detect HEV RNA by a broad-spectrum nested RT-PCR from the fecal and serum samples of the goats that had seroconverted were unsuccessful. In addition, we also attempted to experimentally infect laboratory goats with three well-characterized mammalian strains of HEV but with no success. The results indicate that a HEV-related agent is circulating and maintained in the goat population in Virginia and that the goat HEV is likely genetically very divergent from the known HEV strains.

## **DEDICATION**

I dedicate this dissertation to my wonderful children, Josephine, Sidney, and Adrian.

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## ATTRIBUTIONS

### **Chapter 2: Prior Infection of Pigs with a genotype 3 swine hepatitis E virus (HEV) protects against subsequent challenges with homologous and heterologous genotypes 3 and 4 human HEV.**

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## GENERAL INTRODUCTION

Hepatitis E virus (HEV) is a small, negative strand, non-enveloped RNA virus that is an important cause of acute hepatitis world-wide (Emerson and Purcell, 2003). Feco-oral transmission allows large hepatitis E outbreaks due to contaminated water supplies among human populations where adequate water sanitation is not fully developed (Purdy and Khudyakov, 2011). Sporadic human hepatitis E cases also arise from ingestion of contaminated animal meat products. The case fatality in pregnant women can reach 28% although the overall rate is generally less than 1% (Emerson and Purcell, 2003).

At least four HEV genotypes are currently recognized and the discovery of new animal strains is ongoing (Meng, 2011). HEV genotypes 1 and 2 are restricted to humans while both human and animals strains are found in genotypes 3 and 4. Genetically distinct, currently unclassified HEV strains have been identified in chickens, rats, and fish (Payne et al., 1999; Johne et al., 2010; Batts et al., 2011). Serologic evidence suggests the existence of more HEV strains that have yet to be genetically characterized. HEV is ubiquitous in swine herds, chicken flocks, and some rabbit farms. Clinical signs are not common among pigs and rabbits although chickens exhibit hepatitis and splenomegaly contributing to decreased growth and egg production (Meng et al., 1997; Haqshenas et al., 2001; Cossaboom et al., 2011).

Cross-species infection has been demonstrated experimentally as non-human primates have been infected with genotypes 3 and 4 swine HEV strains and pigs have been infected with genotype 3 and 4 human HEV as well as genotype 3 rabbit HEV

(Arankalle et al., 2006; Meng et al., 1998; Feagins et al., 2008; Cossaboom et al., 2012). Additionally, turkeys were infected with and avian HEV strain isolated from chickens (Sun et al., 2004). Zoonotic infection has been confirmed in cases where undercooked animal meat products were ingested (Tei et al., 2003; Colson et al. 2010).

The discovery of novel strains and the documented zoonotic transmission of certain HEV strains has elevated HEV as a public health concern. Although multiple strains representing various HEV genotypes co-circulate in many regions of the world, little is known about the cross-protective characteristics of homologous or heterologous infections. It is important to continue the search for novel HEV strains and to characterize the cross-protective behavior of HEV infection so that future vaccine design and efficacy may be optimized.

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# **CHAPTER 1**

## **LITERATURE REVIEW**

### **GENERAL HISTORY**

A non-A, non-B enterically-transmitted agent was thought to be responsible for explosive acute hepatitis outbreaks in human populations such as the Delhi epidemics in 1955, since acute markers for hepatitis A virus (HAV) were absent in patients from those outbreaks (Khuroo 1980; Wong, Purcell et al. 1980). The virus responsible for the non-A, non-B enteric hepatitis was genetically identified in 1990 (Reyes, Purdy et al. 1990) and named as hepatitis E virus (HEV). Initially HEV infections were detected in developing countries where sub-optimal sanitation practices exist. At first, diagnosis of HEV was by exclusion when other causes of acute hepatitis were ruled out (Wong, Purcell et al. 1980). Due to progress in education and awareness and the availability of specific diagnostic assays, HEV is currently found in most parts of the world and is associated with individual acute and chronic hepatitis cases as well as the classic, acute explosive outbreak presentation. Many animals are currently known to carry HEV after first being discovered in commercial pigs in 1997 (Meng, Purcell et al. 1997; Haqshenas, Shivaprasad et al. 2001; Zhao, Ma et al. 2009; Johne, Plenge-Bonig et al. 2010; Batts, Yun et al. 2011; Takahashi, Nishizawa et al. 2011).

The disease caused by HEV is indistinguishable from other types of acute hepatitis without advanced serologic or microbiologic diagnoses (Aggarwal 2011). Adults aged 15-40 are most commonly infected and present with clinical signs including jaundice, abdominal pain, and anorexia (Navaneethan, Al Mohajer et al. 2008; Aggarwal

2011). Fecal-oral transmission has also been identified from exposure to contaminated animal meats as well as the classical infection from contaminated water sources (Yazaki, Mizuo et al. 2003; Said, Ijaz et al. 2009; Colson, Borentain et al. 2010). Recent studies have reported chronic hepatitis E cases mostly in immunocompromised individuals (Dalton, Bendall et al. 2009; Nelson, Kmush et al. 2011). HEV infections have also been attributed to some neurologic signs (Peron, Bureau et al. 2007; Kamar, Bendall et al. 2011). The overall mortality of HEV infection is <1% but can reach as high as 28% in infected pregnant women (Purcell 1996; Jilani, Das et al. 2007; Labrique, Zaman et al. 2010).

## **VIROLOGY**

### **Viral Genome**

HEV is a non-enveloped, icosahedral virus that was first visualized in samples of human stools by electron microscopy (Balayan, Andjaparidze et al. 1983). The genome consists of a single-stranded, positive-sense RNA and is approximately 7.2kb in length with a 5' guanosine cap and a 3' polyadenosine tail (Ahmad, Holla et al. 2011). Three open reading frames (ORF), a 5' non-coding region (NCR), and a 3' NCR compose the virus genome (Fig. 1) (Tam, Smith et al. 1991; Tsarev, Emerson et al. 1992; Purcell 1996; Emerson and Purcell 2003).

### **Open Reading Frame 1 (ORF1)**

The first and largest ORF encodes for non-structural proteins and contains putative functional domains required for RNA replication such as a methyltransferase,

papain-like cysteine protease, RNA helicase and a RNA-dependent RNA polymerase (Fig. 1) (Koonin, Gorbalenya et al. 1992; Ahmad, Holla et al. 2011). Certain domains within the ORF 1 are closely related to some other positive-strand RNA viruses like Rubella, alphaviruses, and some coronaviruses (Koonin, Gorbalenya et al. 1992). The presence of the methyltransferase suggests a 5' capping mechanism supported by reports from Emerson et al. that the 5' cap was required for in vivo infection (Emerson, Zhang et al. 2001). As with other RNA viruses, the 5' cap is thought to facilitate recognition of the RNA by the ribosome for translation, and also protection of the virus from the innate immune mechanisms. The functionality of the papain-like-cysteine protease is still unknown but there is again similarity with protease domains from other viruses such as Rubella (Koonin, Gorbalenya et al. 1992). Recent research by Karpe and Lole supported evidence of RNA unwinding activity by the ORF 1 HEV helicase domain as well as its importance for replication of the viral RNA genome (Karpe and Lole 2010a; Karpe and Lole 2010b). The RdRp domain of HEV shares sequence homology with other positive strand RNA viruses as these enzymes are essential for viral genome replication (Koonin, Gorbalenya et al. 1992). An expression product of the RdRp region of ORF 1 was found to associate with the 3' end of genomic HEV RNA and synthesize a complementary strand of viral RNA (Agrawal, Gupta et al. 2001).

### **Open Reading Frame 2 (ORF2)**

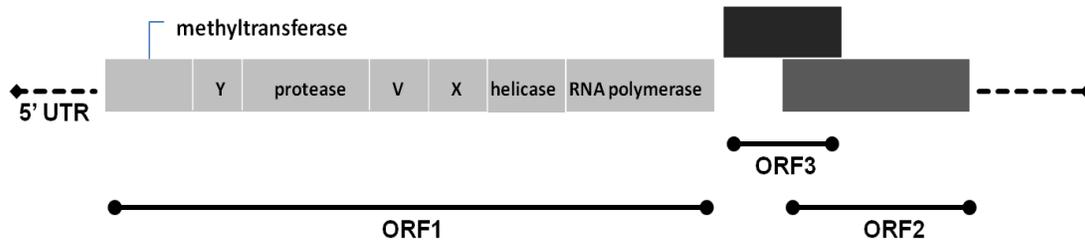
The 660 amino acid, 74 kDa ORF2 protein makes up the capsid protein after dimerization with homologous ORF 2 particles. All well-characterized HEV genotypes appear to share a common epitope within the protruding portion of ORF2 located toward

the C-terminus (Yamashita, Mori et al. 2009; Xing, Wang et al. 2011). Bacterial ORF2 expression generates a product that undergoes proteolytic cleavage to produce a 56 kDa protein capable of forming virus-like particles (VLPs). Three glycosylation sites exist within the ORF2. Studies using both phage display (Schofield, Glamann et al. 2000) and *in vitro* cell culture infectivity (Meng, Dai et al. 2001) assays identified neutralization epitopes within amino acids 578-607 and 452-617, respectively. With this knowledge, recombinant N-terminally truncated ORF2 products have been shown to elicit protective immunity against HEV infection in both non-human primates (Zhang, Emerson et al. 2002; Purcell, Nguyen et al. 2003) and chickens (Guo, Zhou et al. 2006). Human HEV ORF2-based vaccine trials were performed based on the successful completion of those animal studies (Shrestha, Scott et al. 2007; Zhu, Zhang et al. 2010). Both ORF2 and ORF3 proteins are translated from a single, bicistronic subgenomic mRNA (Ahmad, Holla et al. 2011).

### **Open Reading Frame 3 (ORF3)**

The third ORF produces the smallest HEV protein of 123 amino acids. ORF3 has been shown to be necessary for replication and infectivity *in vivo* (Graff, Nguyen et al. 2005; Emerson, Nguyen et al. 2006; Huang, Opriessnig et al. 2007) but not *in vitro* where viral particles from ORF3-null mutant are still able to infect other cells (Emerson, Nguyen et al. 2006). ORF3 protein is thought to be involved in cell signaling and cellular virion release (Yamada, Takahashi et al. 2009; Kenney, Pudupakam et al. 2012) as it is associated with microtubules (Kannan, Fan et al. 2009) and cytoskeletal elements (Zafrullah, Ozdener et al. 1997) as well as endosomes (Chandra, Kar-Roy et al. 2008).

**Figure 1: Genomic organization of the hepatitis E virus.**



### **Replication Strategy**

The complete strategy of HEV replication is not yet known; largely due to the lack of efficient cell culture system (Ahmad, Holla et al. 2011). The majority of HEV replication is proposed based on the genome sequence and analogy to other positive-sense RNA viruses. While improved cell culture systems have recently been identified (Okamoto 2011), most HEV research has been performed using reverse genetic techniques in combination with animal model studies. Non-human primates contribute to research of genotypes 1 and 2 HEV (Emerson, Zhang et al. 2001; Graff, Nguyen et al. 2005) while pigs are excellent models for genotypes 3 and 4 HEV (Huang, Zhang et al. 2008; Pudupakam, Huang et al. 2009). Chickens provide an excellent model for HEV research as well (Billam, LeRoith et al. 2009; Kwon, LeRoith et al. 2011). Hepatocytes appear to be the primary target cell for HEV replication although HEV RNA replicative intermediates have been found in epithelial cells of the gastrointestinal tract and mesenteric lymph nodes (Williams, Kasorndorkbua et al. 2001). Currently, the specific receptor required for initiation of viral entry into the cell is unknown but proteoglycans are thought to be required for attachment to the cell (Kalia, Chandra et al. 2009). The site

for viral uncoating and release within the cell is also not known. Negative-sense genomic RNAs are produced (Panda, Ansari et al. 2000) following immediate production of ORF1 polyprotein. Subgenomic RNAs are translated into ORF2 and ORF3 proteins while genomic RNA is produced directly from the negative-sense templates (Graff, Torian et al. 2006; Huang, Opriessnig et al. 2007).

### **Viral Classification**

HEV belongs to the genus *Hepivirus* within the family *Hepeviridae*. (Meng 2011) At least four recognized genotypes of mammalian HEV have been identified (Ahmad, Holla et al. 2011) worldwide with at least 80% sequence homology within each genotype (Ahmad, Holla et al. 2011). Genotypes 1 and 2 strains of HEV have a limited host range and are restricted to humans, whereas genotypes 3 and 4 have an expanded host range and are zoonotic (Okamoto 2007; Pavio and Mansuy 2010; Pavio, Meng et al. 2010; Meng 2010a; Meng 2010b). Thus far, all viruses identified from pigs worldwide belong to either genotype 3 or genotype 4 (Okamoto 2007; Meng 2010a; Meng 2010b). Besides pigs and humans, HEV has also been genetically identified from chickens (Haqshenas, Shivaprasad et al. 2001), rats (Johne, Plenge-Bonig et al. 2010; Purcell, Engle et al. 2011), mongoose (Nakamura, Takahashi et al. 2006), deer (Tei, Kitajima et al. 2003; Takahashi, Kitajima et al. 2004), rabbit (Zhao, Ma et al. 2009; Cossaboom, Cordoba et al. 2011) and wild trout (Batts, Yun et al. 2011). Two putative new genotypes of mammalian HEV, rat HEV (Johne, Heckel et al. 2010) and a novel wild boar HEV (Takahashi, Nishizawa et al. 2011), have recently been identified. It appears that all mammalian HEV genotypes identified thus far may belong to a single serotype. Given that mammalian and

avian HEV are only approximately 50% identical in nucleotide sequence, it is likely that HEV from chickens belongs to a separate genus (Lu, Li et al. 2006; Bilic, Jaskulska et al. 2009; Marek, Bilic et al. 2010).

## ZOONOSIS

### **Hepatitis E in Pigs**

Hepatitis E is a recognized zoonotic disease, and several animal species such as domestic and wild pigs and deer can serve as reservoirs (Pavio, Meng et al. 2010; Meng 2010a). Swine HEV is enzootic in domestic and wild pigs essentially in all swine-producing countries worldwide and has a very high incidence in swine herds. A large Spanish study that retrospectively analyzed blood samples from more than 20 year ago revealed that 98% of over 200 herds had pigs that were tested positive for HEV antibodies (Casas, Pujols et al. 2009). Additionally, evidence of HEV infection in pigs is found from a great number of geographic regions around the world including the United States (Meng, Purcell et al. 1997), Spain (Casas, Pujols et al. 2009), China (Feng, Zhao et al. 2011; Shen, Ren et al. 2011), New Zeland (Garkavenko, Obriadina et al. 2001), Thailand, Mexico (Cooper, Huang et al. 2005), Japan (Takahashi, Nishizawa et al. 2005) and Brazil (dos Santos, Vitral et al. 2009).

Natural HEV infection of pigs appears to occur at a young age within each herd as the anti-HEV seroprevalence increases with age. Pigs above 4 months of age are generally positive for anti-HEV antibody if HEV infection is prevalent within their herd (Meng, Purcell et al. 1997; Takahashi, Nishizawa et al. 2005). This is reasonable as HEV infection of young pigs would logically occur after maternal, colostral antibody has

waned. The HEV infection in sows within Spanish herds reveals a relatively higher anti-HEV seroprevalence range between 60-70% (Seminati, Mateu et al. 2008). While pigs infected with HEV do not exhibit clinical signs consistent with hepatitis (Meng, Purcell et al. 1997), it is clear that the virus is ubiquitous within commercial swine herds around the world and that HEV appears quite contagious within a pig population (Bouwknegt, Frankena et al. 2008).

### **Swine Exposure and Risk of HEV Infection**

Recent research indicates that HEV isolates within a geographic region, whether from pigs or humans, share similar nucleotide identity (Takahashi, Kitajima et al. 2004; Reuter, Fodor et al. 2009). This data supports HEV zoonosis and, paired with the knowledge that HEV appears to have a broad geographic range, it indicates an elevated infection risk to humans. Research involving humans in regular contact with pigs such as veterinarians, pig handlers, and slaughterhouse staff, suggests that they may be at increased risk of HEV infection (Drobeniuc, Favorov et al. 2001; Meng, Wiseman et al. 2002). Swine associated workers generally had approximately twice the seroconversion rate compared to the general population. In a US study, 27% of veterinarians had IgG anti-HEV antibodies compared to 16% in the surrounding blood donor population (Meng, Wiseman et al. 2002). Approximately 51% of the swine workers in Moldova had IgG anti-HEV compared to 25% in the general population. Additionally, blood donor samples from the general population in US states where swine production is prevalent showed higher prevalence of IgG anti-HEV antibody levels compared individual from non-swine producing locations (Meng, Wiseman et al. 2002).

## **HEV Transmission from Contaminated Animal Meats**

Consumption of HEV infected deer or wild boar meat provide convincing evidence of transmission from animals to humans and is likely the most direct route of infection (Tei, Kitajima et al. 2003; Li, Chijiwa et al. 2005). Three autochthonous cases of acute hepatitis E in France were strongly linked to the ingestion of a popular French sausage made from pig livers (Colson, Borentain et al. 2010). A number of other case studies of acute hepatitis E worldwide contribute strong circumstantial evidence for the direct transmission of HEV to humans through the ingestion of contaminated animal meat products, even though HEV RNA could not be recovered from meats or patients in all cases (Matsuda, Okada et al. 2003; Yazaki, Mizuo et al. 2003; Tamada, Yano et al. 2004; Masuda, Yano et al. 2005). A constant finding in these cases is that the meat is undercooked or traditionally served as raw. Additionally, these cases represent countries where pig livers are commonly consumed or in contact with other meat products. These circumstances likely describe why sporadic, autochthonous meat-born HEV zoonosis are less frequent in the US although infectious HEV RNA is commonly found within pig livers available at local markets (Feagins, Opriessnig et al. 2007). Furthermore, routine cooking protocols are shown to inactivate HEV found in market pig livers, although incubation of contaminated livers for 1 hour at 56C, a temperature equivalent to a medium-to-rare cooking condition in a , could not inactivate HEV (Feagins, Opriessnig et al. 2008).

## **HEV Cross-Species Infection**

Several HEV strains are able to infect multiple animal species under experimental conditions. These studies demonstrate the expanded host ranges of genotypes 3 and 4 HEV while also facilitating the use of new animal models for HEV research (Halbur, Kasorndorkbua et al. 2001), although infected non-human primates nor pigs exhibit consistent clinical signs of acute hepatitis (Meng, Purcell et al. 1997; Aggarwal, Kamili et al. 2001). Chickens may serve as useful animal models as they are a natural hosts to avian HEV that causes a clinical disease known as hepatatitis-splenomegaly syndrome in the US (Haqshenas, Shivaprasad et al. 2001), even though the nucleotide sequence of avian HEV is only 50-60% similar to mammalian HEV strains (Lu, Li et al. 2006). While not capable of infecting non-human primates (Huang, Sun et al. 2004), avian HEV was found to infect turkeys (Sun, Larsen et al. 2004). Although not susceptible to infection with genotype 1 or 2 HEV strains, pigs are natural hosts to both genotype 3 and 4 HEV (Meng, Halbur et al. 1998a) and can be infected by human genotypes 3 and 4 HEV (Meng, Halbur et al. 1998b; Feagins, Opriessnig et al. 2008). Additionally, non-human primates are susceptible to infection by swine genotypes 3 and 4 HEV (Meng, Halbur et al. 1998b; Arankalle, Chobe et al. 2006). Very recently, pigs were experimentally infected with novel rabbit strain of HEV (Cossaboom, Cordoba et al. 2012). As the HEV cellular receptor is not yet identified, the mechanism of host restriction and species specificity are unknown.

## HEV VACCINE

### ORF2 Antigen

The incidence of hepatitis E transmission is greatly reduced with implementation of modern sanitation practices, prevention of contamination of water and meat sources, and proper food preparation. Vaccination of high risk groups also holds promise for HEV prevention. Recombinant polypeptides representing HEV ORF2 capsid proteins are capable of inducing neutralizing antibody when used as vaccines in humans (Shrestha, Scott et al. 2007; Zhu, Zhang et al. 2010), non-human primates (Im, Zhang et al. 2001; Li, Zhang et al. 2005), and chickens (Guo, Zhou et al. 2007). These vaccines utilize N-terminally-truncated ORF2 polypeptides that contain immunogenic epitopes toward the C-terminus of the 660 amino acid protein (Zhou, Purcell et al. 2005; Emerson, Clemente-Casares et al. 2006; Zhang, Dai et al. 2009). Specifically, recombinant proteins containing amino acids 274-660 were most capable of eliciting antibodies in mice (Meng, Dai et al. 2001) and macaques (Zhou, Purcell et al. 2005) compared to other segments of the ORF2 protein, while immunodominant epitopes were found in similar amino acid regions of avian HEV (Haqshenas, Huang et al. 2002; Dong, Zhao et al. 2011). Additionally, when the 111 N-terminal amino acids are absent, HEV virus-like particles (VLPs) are formed (Li, Yamakawa et al. 1997; Li, Takeda et al. 2005) which may provide significant benefit for HEV vaccine efficacy as well as presentation of antigenic epitopes to mucosal surfaces (Niikura, Takamura et al. 2002; Li, Takeda et al. 2005; Xing, Wang et al. 2011). Truncated and full-length recombinant ORF2 proteins have also been extensively utilized for the detection of HEV antibodies via serological assays, which are an integral part of HEV diagnostics (Tsarev, Tsareva et al. 1993; He, Ching et

al. 1995; Meng, Purcell et al. 1997; Ghabrah, Tsarev et al. 1998; Anderson, Li et al. 1999).

### **HEV Cross-protection**

HEV ORF2 antigens are capable of cross-reacting with antibodies from heterologous HEV strains (Engle, Yu et al. 2002; Haqshenas, Huang et al. 2002; Cossaboom, Cordoba et al. 2012) while convalescent sera from animals infected with any of the four HEV genotypes was capable of neutralizing cells infected with genotype 1 HEV (Emerson, Clemente-Casares et al. 2006). Cross-protection due to previous infection with homologous or heterologous strains of HEV was demonstrated in rhesus macaques (Huang, Zhang et al. 2008) and pigs (Sanford, Dryman et al. 2011). Thus far, HEV ORF2 antigen vaccination has not been studied in pigs.

### **Current Human HEV Vaccine Trials**

Recently, a comprehensive HEV vaccine trial consisting of over 100,000 participants was performed in China utilizing a recombinant ORF2 protein representing amino acids 368-606. Each vaccine dose contained 30µg of the purified antigen adsorbed with aluminum hydroxide. Three doses of vaccine were given at 0, 1, and 6 months of to participants of mixed sex and aged 16-65 years. Participants who received 3 doses of vaccine were completely protected from HEV infection. Additionally, very few vaccine-related adverse reactions were recorded, even from pregnant women, indicating excellent safety as well as efficacy (Zhu, Zhang et al. 2010). These results are extremely promising. Future studies involving high risk individuals such as pregnant women and

immunocompromised patients are needed before the use of the vaccine in these populations. In a smaller, 5300 participant Nepalese study, another recombinant HEV ORF2 vaccine was also administered in 3 doses with similar results. Over 95% of the vaccinates were protected from HEV infection and the rate of vaccine-related adverse reaction was very low (Shrestha, Scott et al. 2007). The results of these two vaccine trials show promise for the full-scale production and use of HEV vaccine and that recombinant ORF2 products are ideal candidates.

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## **CHAPTER 2**

### **Prior infection of pigs with a genotype 3 swine hepatitis E virus (HEV) protects against subsequent challenges with homologous and heterologous genotypes 3 and 4 human HEV**

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#### **ABSTRACT**

Hepatitis E virus (HEV) is an important human pathogen. At least four recognized and two putative genotypes of mammalian HEV have been reported: genotypes 1 and 2 are restricted to humans whereas genotypes 3 and 4 are zoonotic. The current experimental vaccines are all based on a single strain of HEV, even though multiple genotypes of HEV are co-circulating in some countries and thus an individual may be exposed to more than one genotype. Genotypes 3 and 4 swine HEV is widespread in pigs and known to infect humans. Therefore, it is important to know if prior infection with a genotype 3 swine HEV will confer protective immunity against subsequent exposure to genotypes 3 and 4 human and swine HEV. In this study, specific-pathogen-free pigs were divided into 4 groups of 6 each. Pigs in the three treatment groups were each inoculated with a genotype 3 swine HEV, and 12 weeks later, challenged with the same genotype 3 swine HEV, a genotype 3 human HEV, and a genotype 4 human HEV, respectively. The

control group was inoculated and challenged with PBS buffer. Weekly sera from all pigs were tested for HEV RNA and IgG anti-HEV, and weekly fecal samples were also tested for HEV RNA. The pigs inoculated with swine HEV became infected as evidenced by fecal virus shedding and viremia, and the majority of pigs also developed IgG anti-HEV prior to challenge at 12 weeks post-inoculation. After challenge, viremia and fecal virus shedding of challenge viruses were not detected, suggesting that prior infection with a genotype 3 swine HEV prevented pigs from developing viremia and fecal virus shedding after challenges with homologous and heterologous genotypes 3 and 4 HEV. The results from this study have important implications for future development of an effective HEV vaccine.

**Keywords:** hepatitis E virus (HEV); swine HEV; cross-protection; prior infection; pigs

## INTRODUCTION

Hepatitis E virus (HEV), the causative agent of human hepatitis E, is an important pathogen worldwide (Meng, 2010a, 2010b; Purcell and Emerson, 2008). Although the mortality rate is generally low, it can reach up to 28% in HEV-infected pregnant women (Bhatia et al., 2008; Jilani et al., 2007). As a fecal-orally transmitted virus, contaminated water is the most common source of infection for large outbreaks in developing countries due to poor sanitation conditions (Emerson and Purcell, 2003; Meng, 2010b). In some industrialized countries, sporadic cases of acute hepatitis E that are likely caused by zoonotic transmission have also been reported. Cases of autochthonous HEV infections from industrialized countries are increasing (Aikawa et al., 2002; Colson et al., 2010; Jameel, 1999; Legrand-Abravanel et al., 2010; Mizuo et al., 2005).

At least four recognized genotypes of mammalian HEV have been identified worldwide: genotypes 1 and 2 strains of HEV have a limited host range and are restricted to humans, whereas genotypes 3 and 4 have an expanded host range and are zoonotic (Meng, 2010a, 2010b; Pavio et al., 2010; Pavio and Mansuy, 2010). The first animal strain of HEV, swine HEV, was identified from pigs in the United States (Meng et al., 2007). Thus far, all viruses identified from pigs worldwide belong to either genotype 3 or genotype 4 (Meng, 2010a, 2010b; Okamoto, 2007). Besides pigs and humans, HEV has also been genetically identified from chickens (Haqshenas et al., 2001), rats (Johne et al., 2010), mongoose (Nakamura et al., 2006), deer (Takahashi et al., 2004; Tei et al., 2003), and rabbits (Zhao et al., 2009). Two putative new genotypes of mammalian HEV, rat HEV (Johne et al., 2010) and a novel wild boar HEV (Takahashi et al., 2011), have recently been identified. It appears that all mammalian HEV genotypes identified thus far

may belong to a single serotype (Emerson and Purcell, 2003; Meng, 2010b), since antigenic cross-reactivity of the capsid protein among mammalian HEV strains and between avian HEV and mammalian HEV have been reported (Haqshenas et al., 2002). The avian HEV from chickens likely belongs to a separate genus (Bilic et al., 2009).

Hepatitis E is a recognized zoonotic disease, and several animal species such as domestic and wild pigs and deer can serve as reservoirs (Meng, 2010b; Pavio et al., 2010). Swine HEV is enzootic in domestic and wild pigs essentially in all swine-producing countries worldwide and has a very high incidence in swine herds (Bouwknegt et al., 2008; Casas et al., 2009; De Deus et al., 2008a, 2008b; Sonoda et al., 2004; Van der Poel et al., 2001). It has been reported that humans in regular contact with pigs such as veterinarians and other pig handlers appear to be at higher risk of HEV infection (Drobeniuc et al., 2001; Meng et al., 2003). Under experimental conditions, genotypes 3 and 4 strains of human HEV infected pigs, and conversely genotypes 3 and 4 strains of swine HEV infected non-human primates (Arankalle et al., 2006; Halbur et al., 2001; Meng et al., 1998). Strains of HEV recovered from human hepatitis E patients are genetically indistinguishable from swine HEV recovered from pig livers in grocery stores, and zoonotic human infections with genotypes 3 and 4 swine HEV through direct contact with infected animals or via consumption of undercooked or raw animal meats have also been reported (Colson et al., 2010; Rolfe et al., 2010; Takahashi et al., 2004; Yazaki et al., 2003).

Currently, there is very limited data regarding the cross-protection between different HEV strains belonging to different genotypes. The objective of this study was to evaluate the cross-protective ability of prior genotype 3 swine HEV infection in pigs

against subsequent challenge with homologous swine HEV strain and heterologous human HEV strains of different genotypes.

## **MATERIALS AND METHODS**

### **Virus Stocks**

The HEV infectious stocks used in this study were generated from previous studies, including a genotype 3 swine HEV with an infectious titer of  $10^{4.5}$  50% pig infectious dose (PID<sub>50</sub>) (Halbur et al., 2001; Meng et al., 1988), a genotype 3 U.S. strain of human HEV (Feagins et al., 2008; Halbur et al., 2001; Meng et al., 1988), and a genotype 4 Taiwanese strain of human HEV each diluted to approximately  $3.4 \times 10^8$  genomic equivalent (GE) titer per ml of 10% fecal suspension in PBS buffer (Feagins et al., 2008; Wu et al., 2000). These virus stocks are known to be infectious when intravenously inoculated into pigs (Feagins et al., 2008; Halbur et al., 2001; Meng et al., 1988).

### **Experimental Design**

Twenty-four, 8-week-old, cross-bred specific-pathogen-free (SPF) pigs that were tested negative for IgG anti-HEV were randomly divided into 4 groups of 6 pigs each. Pigs in each group were housed in separate rooms within a climate controlled Biosafety Level 2 (BSL-2) facility. A strict biosecurity protocol was followed for feeding and sample collection. Group 1 negative control pigs were inoculated intravenously (IV) with sterile PBS buffer and subsequently challenged with PBS buffer at 12 week post-inoculation (wpi). Pigs in groups 2, 3, and 4 were all inoculated IV with  $10^{4.5}$  PID<sub>50</sub> of a

genotype 3 strain of swine HEV, and subsequently challenged, at 12 wpi, with  $3.4 \times 10^8$  GE titer of a genotype 3 US-2 strain of human HEV,  $10^{4.5}$  PID<sub>50</sub> titer of the same genotype 3 strain of swine HEV used for initial inoculation, and  $3.4 \times 10^8$  GE titer of a genotype 4 Taiwanese strain of human HEV, respectively. At 4 weeks post-challenge (wpc), all pigs were necropsied. Serum and fecal samples were collected prior to inoculation and weekly thereafter, and tested for the presence of HEV RNA by nested RT-PCR. The weekly serum samples were also tested for IgG anti-HEV with an ELISA.

### **ELISA for the detection of IgG anti-HEV**

The ELISA was performed essentially as previously described (Feagins et al., 2007, 2008; Halbur et al., 2001; Meng et al., 1997, 1998) with the exception that the antigen used in this study was a recombinant protein expressed and purified from *E.Coli*, representing amino acids 452-617 of the HEV capsid protein (GenWay, Inc, San Diego, CA). The ELISA cutoff was set at 3 standard deviations above the mean OD values of the pre-inoculation samples (Feagins et al., 2007, 2008; Halbur et al., 2001; Meng et al., 1997, 1998).

### **Nested RT-PCR to detect HEV RNA in fecal and serum samples**

The nested RT-PCR assays used for the detection of HEV RNA from serum and fecal samples were essentially the same as previously described (Feagins et al., 2007, 2008) with slight modifications. Strain-specific primers to amplify the ORF2 region of each virus were designed to detect the genotype 3 swine HEV, genotype 3 US-2 human HEV, and genotype 4 Taiwanese human HEV, respectively. The nested RT-PCR primer

sequences for the detection of genotype 3 swine HEV are: first round, forward [5'-AGCTCCTGTACCTGATGTTGACTC-3'], reverse [5'-CTACAGAGCGCCAGCCTTGATTGC-3']; second round, forward [5'-GCTCACGTCATCTGTCGCTGCTGG-3'], and reverse [5'-GGGCTGAACCAAATCCTGACATC-3']. The primer sequences for the detection of genotype 3 US-2 strain of human HEV include: first round, forward [5'-GTTGCTCTTCGTGCTTTTGCCTATG-3'], and reverse [5'-CCAGCGGGCGATAACGGATTGTAGC-3']; second round, forward [5'-GGCGGTGGTTTCTGGGGTGAC-3'], and reverse [5'-GTACCCGAAGCGACAGATGACG-3']. The primers for the detection of genotype 4 Taiwanese strain of human HEV consist of: first round, forward [5'-CGCAGGTTTATGGTGTAGCC-3'], and reverse [5'-GTCGGGGTGGTGGTTGTTTGGG-3']; second round, forward [5'-GCTCCTCCTGCTTTTGCCTATGC-3'], and reverse [5'-CGGTGGCGATGGTGGATGTGAGTG-3'].

Total RNAs were extracted with Trizol Reagent (Life Technologies, Carlsbad, CA) from 150 µl of serum or 10% fecal suspension. Reverse transcription and cDNA synthesis were performed at 42°C for 1 hr with 1 µl (10 µM) of the first round reverse primer specific for each virus, 1 µl (200 units/µl) of Superscript II reverse transcriptase (Life Technologies, Carlsbad, CA), 1 µl of 0.1M dithiothreitol, 4 µl of 5x RT buffer, 0.5 µl (40 units/µl) of RNasin ribonuclease inhibitor (Promega, Madison, WI), and 1 µl of 10 mM deoxynucleoside triphosphates. Nested PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems, Carlsbad, CA) and genotype-specific primers was then performed

on each sample [34]. The cycling parameters included an initial denaturation step at 95°C for 9 min, followed by 39 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 52°C, extension for 1 min at 72°C, and a final extension step at 72°C for 7min. The expected final PCR products of the amplified ORF2 gene region for genotype 3 swine HEV, genotype 3 human HEV, and genotype 4 human HEV are 680 bp, 779 bp, and 508 bp, respectively.

## **RESULTS**

### **Fecal virus shedding in pigs inoculated with genotype 3 swine HEV prior to challenge**

Rectal temperature, body weight or feed intake was not recorded, since HEV infection in pigs does not cause clinical sign of diseases (Halbur et al., 2001). Three pigs in the negative control group 1 and one pig in group 4 died of over-eating prior to challenge, and were excluded from the study. Prior to challenge, the weekly fecal samples from each pig were tested for the presence of the genotype 3 swine HEV by a nested RT-PCR. Fecal virus shedding started at 1 wpi with five positive pigs in group 2, three positive pigs in group 3, and 2 positive pigs in group 4 (Table 1). Genotype 3 swine HEV RNA was thereafter detected in the feces of all pigs in groups 2, 3 and 4 prior to challenge at 12 wpi (Table 1). Fecal virus shedding was not detectable in any of the group 1 pigs inoculated with PBS buffer (Table 1).

### **Viremia in pigs inoculated with genotype 3 swine HEV prior to challenge**

During the 12 weeks prior to challenge, viremia was detected variably in 16 of 17 pigs (except for pig # 1644) inoculated with the genotype 3 swine HEV in groups 2, 3, and 4 (Table 2). Viremia began at 1 wpi in 4/6 pigs in group 2, 4/6 pigs in group 3, and 2/5 pigs in group 4 (Table 2). Negative control pigs in group 1 inoculated with PBS buffer remained negative (Table 2). The viremia in most pigs is transient lasting 1-3 weeks (Table 2), however some animals such as pigs #1738, #1744 and #1748 in group 2, and pig #1640 in group 4 had a prolonged period of viremia (Table 2).

### **Seroconversion to IgG anti-HEV in pigs inoculated with genotype 3 swine HEV prior to challenge**

Seroconversion to IgG anti-HEV started at 1 wpi in some of the groups 2 and 4 pigs, and at 2 wpi in some of the group 3 pigs (Fig. 1). During the first 12 weeks prior to challenge, pigs in all three experimental groups seroconverted to IgG anti-HEV antibody except for pig 1645 in group 3 and pig #1740 in group 4 (Fig. 1). Negative control pigs (ID# 1739 and #1746) remained seronegative throughout the study, although pig #1737 had an elevated OD reading that was at the borderline of the cutoff (Fig. 1). Serum and fecal samples from pig#1737 are tested negative by RT-PCR for HEV RNA throughout the study.

## **Protection and cross-protection of HEV infections in pigs challenged with homologous and heterologous genotypes of HEV**

At 12 wpi, pigs in groups 1, 2, 3, and 4 were challenged with sterile PBS buffer (control), a genotype 3 US-2 strain of human HEV, the same genotype 3 strain of swine HEV as used in the inoculum, and a genotype 4 Taiwanese strain of human HEV, respectively. After challenge, weekly serum and fecal samples were collected from each pig, and all pigs were necropsied at 4 wpc. Fecal virus shedding and viremia were tested by nested RT-PCR assays specific for each challenge strain of HEV. At no time point after challenge, was there any HEV RNA detected in the sera of pigs in any group (Table 2). Likewise, HEV RNA specific to the challenge virus was not detected in the feces of pigs in group 2 or group 4. Pig 1649 in group 4 continued to shed genotype 3 swine HEV from the original inoculation as confirmed by sequence analysis in feces for one week after challenge (Table 1). In group 3 pigs challenged with the homologous same genotype 3 strain of swine HEV, pig 1645 shed the same genotype 3 swine HEV in feces at 1 wpc while pig 1650 shed the same genotype 3 swine HEV in feces at 4 wpc (Table 1). After challenge, the levels of IgG anti-HEV antibodies maintained in similar levels with a slight decrease in titers in some pigs towards the end of the study. A booster effect on IgG anti-HEV level after challenge was not evident, which is expected since the prior infection protected against the challenge (Fig. 1). As expected, viremia, fecal virus shedding and seroconversion were not detected in the negative control pigs in group 1 (Fig. 1).

## DISCUSSION

A vaccine against HEV has not yet been available, largely due to the inability to efficiently propagate HEV in cell culture. The recent adaptation of several strains of HEV to replicate more efficiently in established cell lines (Okamoto, 2011; Shukla et al., 2011) may aid the future development of an affordable HEV vaccine. The current experimental HEV vaccines, which are all based on the recombinant ORF2 capsid protein of a single strain of HEV, are promising in clinical trials (Arankalle et al., 2009; Shrestha et al., 2007; Zhu et al., 2010), however their efficacies against the diversified field strains of HEV from different genotypes, especially against the emerging and zoonotic strains of HEV, need to be evaluated. In a given geographic region, multiple genotypes of HEV are co-circulating in the same population. For example, genotypes 1, 3 and 4 HEV strains are all circulating in China (Zhang et al., 2010). Thus, it is possible that an individual may be exposed to more than one genotype of HEV. It will be important to know if prior exposure to one genotype will induce sufficient protective immunity against subsequent exposure to a different genotype. In addition, the high prevalence of swine HEV infection in pigs increases the risk of zoonotic human infections by swine HEV (Meng et al., 2003). For example, individuals from the major swine-producing state of Minnesota are at least 5 times more likely to be seropositive for IgG anti-HEV than individuals from traditionally non-major swine state such as Alabama (Meng et al., 2003). It is unclear if the individuals infected by the zoonotic swine HEV will confer protection against subsequent exposure to human strains of HEV of a different genotype. This study was designed to partially address these questions by evaluating the cross-protection of HEV infection in pigs inoculated with a genotype 3 swine HEV and then subsequently

challenged with homologous genotype 3 swine HEV and heterologous human HEV strains from a different genotype.

All experimental pigs inoculated with the genotype 3 swine HEV became infected as evidenced by seroconversion to IgG anti-HEV (Fig. 1), fecal virus shedding (Table 1) and viremia (Table 2). Consistent with previous infection studies in pigs, the inoculated animals remained clinically healthy but began to shed virus in feces and become viremic within the first two weeks after inoculation. As was the case in previous studies, pig-to-pig variations in the onset and duration of fecal virus shedding, viremia and seroconversion were observed in inoculated pigs. Some inoculated animals such as pigs #1738 and #1748 in group 2 and pigs #1640 and #1649 in group 4 had extended fecal virus shedding and prolonged viremia. The exact reason for the observed long period of fecal virus shedding and viremia is not known, but similar observations have been reported in some of HEV-infected chickens and pigs in our previous studies (Billam et al., 2005; Feagins et al., 2007, 2008). Also, cases of chronic HEV infections and prolonged virus shedding have been reported in transplant patients as well HIV-infected individuals (Legrand-Abravanel et al., 2010; Renou et al., 2010). In addition, long-term shedding of genotype 3 swine HEV for up to 12 weeks in naturally-infected pigs has also been reported (Kanai et al., 2010). It is possible that pigs infected by HEV may develop persistent infection more often than what we originally thought. While this study is not designed to determine the reason for the observed pig-to-pig variation in virus shedding or viremia, it is possible that some behavioral characteristics such as stress or altered sleep patterns in a certain group or animal may affect the course of HEV infection. There is also a possibility that some animals with extended fecal virus shedding and viremia

may become re-infected before the animal mounts an effective immune response from the initial infection.

Importantly for this study, pigs inoculated with the genotype 3 swine HEV seroconverted to IgG anti-HEV antibodies (Fig. 1). Pig-to-pig variations in IgG anti-HEV antibody response are evident within each group. A few pigs such as pig #1645 in group 3 and pig #1740 in group 4 had a very low level of IgG anti-HEV response below the cutoff before challenge. However, overall the majority of pigs inoculated with swine HEV developed IgG anti-HEV prior to challenge at 12 wpi, and the results indicated that pigs inoculated with the swine HEV became infected and elicited humoral immune response prior to challenge.

At 12 wpi, the pigs were challenged with two different genotypes of human HEV (genotypes 3 and 4) as well as with the homologous same genotype 3 strain of swine HEV. After challenge, fecal virus shedding and viremia were undetectable in the pigs that had been previously infected with the genotype 3 swine HEV. Two pigs in group 3 (ID# 1645, # 1650) and one pig in group 4 (ID# 1649) had transient fecal virus shedding of one week after challenge, and subsequent sequence analyses of the excreted viruses recovered from the fecal samples of the three pigs indicated that the virus belongs to the genotype 3 swine HEV from the original inoculation. Thus, the transient one-week fecal virus shedding after challenge in pig #1649 in group 4 is due to the persistent infection of the genotype 3 swine HEV from the original inoculation rather than from the challenge genotype 4 human HEV. However, the source of genotype 3 swine HEV detected after challenge in pigs #1645 and #1650 in group 3 could not be determined since the pigs in this group were challenged with the same genotype 3 swine HEV. It is possible that the

transient one-week fecal virus shedding in these two pigs may be due to incomplete protection against the homologous challenge virus, since fecal virus shedding from the initial inoculation were fully cleared in both pigs by 6 weeks post-inoculation. Overall, the results from this study clearly demonstrated that prior infection with the genotype 3 swine HEV prevented pigs from developing viremia and fecal virus shedding upon challenges with homologous swine HEV and heterologous human HEV strains. Our result is consistent with a non-human primate study in which rhesus macaques initially infected with genotypes 1 or 4 human HEV are protected from subsequent challenge with heterologous genotypes of HEV (Huang et al., 2008). The results from this study suggest that a vaccine derived from one genotype of HEV or from an animal strain of HEV is sufficient to elicit protective immunity against other HEV genotypes, and this finding is important when designing future HEV vaccine strategies.

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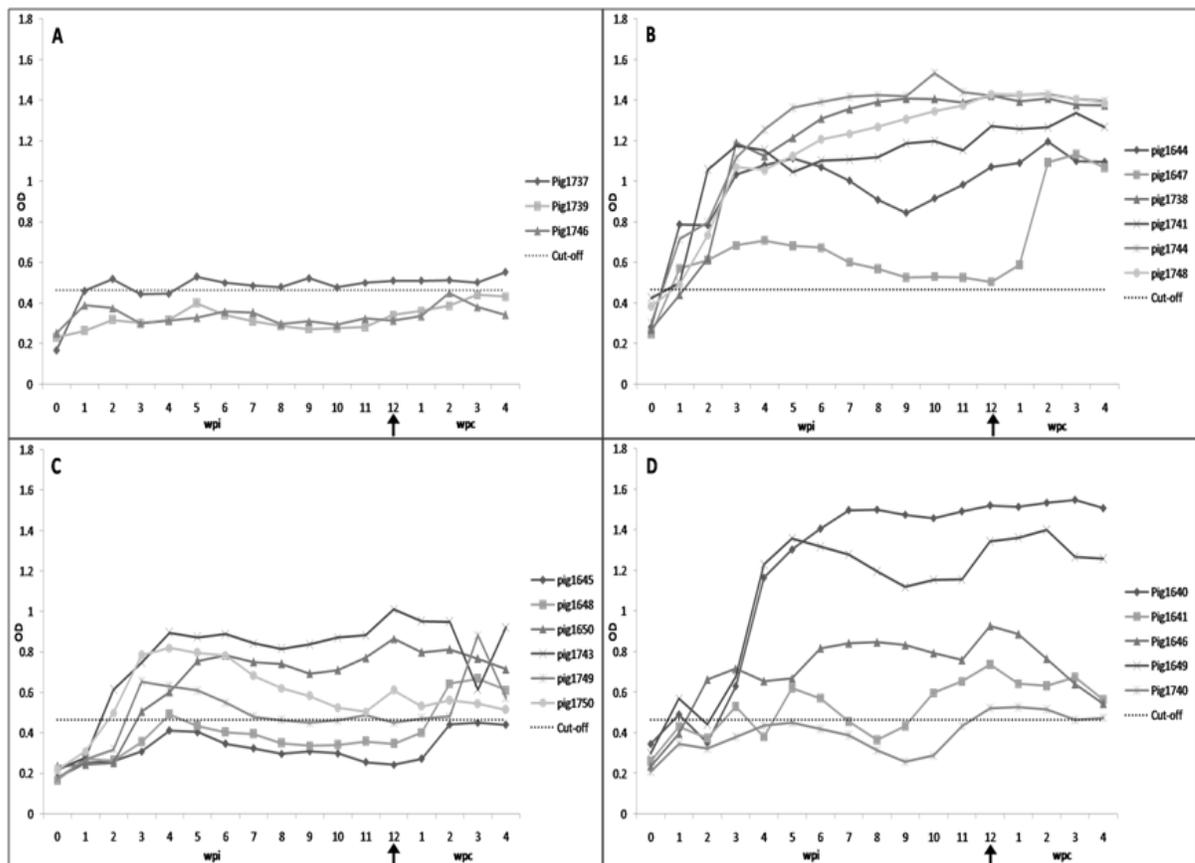
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## Figure Legend

**Figure 1.** IgG anti-HEV response in pigs inoculated with a genotype 3 swine HEV and subsequently challenged, at 12 weeks post-inoculation (wpi), with different strains of human and swine HEV. **A:** Pigs inoculated and challenged with PBS buffer. **B:** Pigs inoculated with a genotype 3 swine HEV and challenged at 12 wpi with a genotype 3 human HEV (US-2 strain). **C:** Pigs inoculated with a genotype 3 swine HEV and challenged at 12 wpi with the same genotype 3 swine HEV. **D:** Pigs inoculated with a genotype 3 swine HEV and challenged at 12 wpi with a genotype 4 human HEV (Taiwanese strain). Arrow in the X-axis indicates the time of challenge at 12 wpi. All pig were necropsied at 4 weeks post-challenge (wpc).



**Table 1.** Detection of fecal virus shedding by RT-PCR in pigs inoculated with a genotype 3 strain of swine HEV and subsequently challenged at 12 weeks post-inoculation (wpi) with different genotypes of human and swine HEV

Groups	Pig ID#	Inoculum	Weeks post-inoculation (wpi)												Challenge inoculum	Weeks post-challenge (wpc)							
			1	2	3	4	5	6	7	8	9	10	11	12		1	2	3	4				
<b>1</b>		<b>PBS buffer</b>														<b>PBS buffer</b>							
	1737		-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	
	1739		-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	
	1746	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
<b>2</b>		<b>Genotype 3 swine HEV</b>														<b>Genotype 3 human HEV</b>							
	1644		+	+	+	+	+	+	+	+	+	-	-	-	-		-	-	-	-	-		
	1647		-	+	+	-	+	+	+	+	+	-	-	-	-		-	-	-	-	-	-	
	1738		+	+	+	-	+	+	-	+	+	-	-	-	-		-	-	-	-	-	-	
	1741		+	+	+	+	+	+	+	+	+	-	-	-	-		-	-	-	-	-	-	
	1744		+	+	+	-	+	+	+	+	+	-	-	-	-		-	-	-	-	-	-	
	1748	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-			
<b>3</b>		<b>Genotype 3 swine HEV</b>														<b>Genotype 3 swine HEV</b>							
	1645		-	+	-	-	-	-	-	-	-	-	-	-	-		-	+	*	-	-	-	
	1648		-	+	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	
	1650		+	+	+	+	+	-	-	-	-	-	-	-	-		-	-	-	-	-	+	*
	1743		+	+	+	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-
	1749		+	+	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-
	1750	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
<b>4</b>		<b>Genotype 3 swine HEV</b>														<b>Genotype 4 human HEV</b>							
	1640		-	+	+	+	+	+	+	+	+	+	+	+	+		-	-	-	-	-	-	
	1641		+	+	+	+	+	+	+	+	+	+	+	+	+		-	-	-	-	-	-	
	1646		-	+	-	-	+	+	+	+	+	+	+	+	+		-	-	-	-	-	-	
	1649		-	+	-	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	
	1740	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-			

\*Positive for genotype 3 swine HEV RNA but negative for genotype 4 human HEV RNA

**Table 2.** Detection of viremia by RT-PCR in pigs inoculated with a genotype 3 strain of swine HEV and subsequently challenged at 12 weeks post-inoculation (wpi) with different genotypes of human and swine HEV

Groups	Pig ID#	Inoculum	Weeks post-inoculation (wpi)												Challenge	Weeks post-challenge (wpc)				
			1	2	3	4	5	6	7	8	9	10	11	12		1	2	3	4	
<b>1</b>		<b>PBS buffer</b>														<b>PBS buffer</b>				
	1737		-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
	1739		-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
	1746		-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
<b>2</b>		<b>Genotype 3 swine HEV</b>														<b>Genotype 3 human HEV</b>				
	1644		-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
	1647		+	+	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
	1738		-	+	+	+	+	-	+	+	+	-	-	-	-		-	-	-	-
	1741		+	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
	1744		+	+	+	+	+	+	+	-	-	-	-	-	-		-	-	-	-
	1748		+	+	+	+	+	+	-	-	+	+	-	-	-		-	-	-	-
<b>3</b>		<b>Genotype 3 swine HEV</b>														<b>Genotype 3 swine HEV</b>				
	1645		+	+	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
	1648		+	+	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
	1650		-	+	+	-	-	-	-	-	-	-	-	-	-		-	-	-	-
	1743		-	+	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
	1749		+	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
	1750		+	+	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
<b>4</b>		<b>Genotype 3 swine HEV</b>														<b>Genotype 4 human HEV</b>				
	1640		+	-	+	+	+	+	+	+	+	+	+	+		-	-	-	-	
	1641		-	+	+	-	-	-	-	-	-	-	-	-		-	-	-	-	
	1646		+	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	
	1649		-	+	+	-	+	+	-	-	-	-	-	-		-	-	-	-	
	1740		-	-	-	-	-	-	-	-	-	+	-	-		-	-	-	-	

## CHAPTER 3

### **Assessment of the cross-protective capability of recombinant capsid proteins derived from pig, rat, and avian hepatitis E viruses (HEV) against challenge with a genotype 3 HEV in pigs**

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#### **ABSTRACT**

Genotypes 3 and 4 hepatitis E virus (HEV) infect many species globally while genotypes 1 and 2 appear restricted to humans. HEV is an important public health concern due largely to water-borne outbreaks but recent research confirms individual cases of zoonotic transmission due to human exposure to contaminated animal meats. Despite the diverse range of HEV infection, all genotypes belong to a single serotype. Immunogenic epitopes are located within the open reading frame 2 (ORF 2) capsid protein and recombinant ORF 2 antigens are capable of preventing HEV infection in non-human primates and chickens. In this current study we expressed and purified N-truncated ORF 2 antigens based on swine, rat, and avian HEV strains. Thirty pigs were randomly divided into groups of 6 pigs each and initially vaccinated with 200µg swine ORF 2 antigen, rat ORF 2 antigen, avian ORF 2 antigen, or PBS buffer (positive and negative control groups) and boosted with the same vaccine 2 weeks later. At 4 wks,

after confirming seroconversion to IgG anti-HEV antibody with ELISA, all groups except the negative control were challenged with swine genotype 3 HEV (administered intravenously). The protective and cross-protective abilities of these antigens were determined following swine genotype 3 challenge by evaluating both serum and fecal samples for HEV RNA using nested RT-PCR.

## INTRODUCTION

Hepatitis E virus (HEV), the causative agent of the feco-orally transmitted acute hepatitis E in humans, is an important pathogen worldwide [1-5]. The mortality rate can reach up to 28% in HEV-infected pregnant women, although the mortality is less than 1% in the general population [6, 7]. Large acute hepatitis E outbreaks due to contaminated water sources occur in countries where modern sanitation practices are not commonly implemented [4, 8]. While less common, zoonotic transmissions leading to sporadic or cluster cases of acute hepatitis E are a growing concern as they can occur in both industrialized and developing countries [9]. Risk of potential HEV zoonosis becomes more significant with discovery of novel HEV strains in numerous animal species around the world [10-19]. Thus far, at least four recognized and two putative genotypes of mammalian HEV have been identified worldwide. The genotypes 1 and 2 HEV infect only humans, while genotypes 3 and 4 HEV have an expanded host range and are zoonotic [3-5, 9].

Meng et al (1997) identified the first animal strain of HEV in pigs designated as swine HEV, while a strain of HEV from chickens designated avian HEV was isolated in 2001 [20, 21]. HEV infection in pigs appears to be subclinical [20], and is ubiquitous within swine herds worldwide [22-28]. All swine HEV strains currently identified worldwide belong to either genotype 3 or genotype 4 [3, 4, 29] except for a putative new genotype recently isolated from a wild boar in Japan [30]. Genotypes 3 and 4 HEV are able to infect across species [3-5, 31]. Additionally, a genotype 3 strain of HEV isolated from rabbits was capable of infecting pigs [32]. Confirmed human infections due to zoonotic transmissions arise primarily from consumption of contaminated pig liver, pork

products such as pig liver sausage, and deer meats [17, 33] as these types of products are shown to be positive for infectious HEV [34].

Avian HEV is enzootic within chicken flocks and is identified as the cause of hepatitis-splenomegaly syndrome (HS syndrome) [35]. Thus far at least three genotypes of avian HEV have been identified from chickens worldwide including the United States, Australia, Spain, China etc [36-39]. Avian HEV only shares 50-60% nucleotide sequence identity with swine or human HEV strains and likely belongs to a separate genus [21, 36, 37]. Besides pigs, humans, and chickens, HEV has also been genetically identified from rats [14, 19], mongoose [40], deer [17, 41], rabbits [42, 43], and even trout [10]. All mammalian HEV genotypes identified thus far belong to a single serotype.

Hepatitis E transmission is greatly reduced with implementation of modern sanitation practices, prevention of contamination of water and meat sources, and proper food preparation. Vaccination of high risk groups also holds great promise for HEV prevention and control. Recombinant capsid proteins of HEV are capable of inducing neutralizing antibodies when used as candidate vaccines in humans [44, 45], non-human primates [46, 47], and chickens [48]. These candidate vaccines utilize N-terminally truncated capsid proteins that contain immunogenic epitopes toward the C-terminus of the 660 amino acid protein [49]. Additionally, when the 111 N-terminal amino acids are absent, the truncated capsid protein forms virus-like particles (VLPs) [50, 51] which may provide significant benefit for HEV vaccine efficacy [50, 52, 53]. HEV capsid antigens are capable of cross-reacting with antibodies from heterologous HEV strains [32, 54, 55] while convalescent sera from animals infected with any of the four recognized HEV genotypes were capable of neutralizing genotype 1 HEV in vitro [49]. In addition, cross-

protections due to prior infection against homologous or heterologous strains of HEV were demonstrated in rhesus macaques [56] and pigs [57]. The objective of this study was to determine if the recombinant capsid antigens derived from swine, rat, and avian HEV strains can induce cross-protection against a genotype 3 HEV challenge in a pig model.

## **MATERIALS & METHODS**

### **Expression and purification of truncated recombinant capsid proteins derived from swine, rat, and avian HEV strains**

The N-terminal truncated capsid proteins from swine, rat, and avian sources were expressed in bacterial expression system to represent the immunogenic ORF2 capsid proteins lacking the 111 N-terminal amino acid residues [50, 58-60]. Briefly, the truncated ORF2 genes were amplified from respective plasmids that contained avian, genotype 3 swine, and rat HEV ORF2 genes with strain-specific primers (Table 1). Each amplified PCR products was gel-purified, digested with BamHI and BglII restriction enzymes, and subsequently cloned into the pRSET-A bacterial expression vector (Invitrogen). The authenticity of each ORF2 gene insert was verified by sequencing and sequence analyses of the recombinant plasmids. Each recombinant pRSET-A plasmid was transformed into BL21(DE3)pLysS (Novagen) *E. coli* cells and then grown in an auto-inducing translation system using Overnight Express Instant TB Medium (Novagen) essentially as described [61]. To facilitate the purification of the resulting recombinant capsid proteins, six histidine residues were incorporated into the proteins.

To purify the expressed recombinant capsid proteins, bacterial cell pellets were lysed with BugBuster Protein Extraction Reagent (Novagen) mixed with 6M urea as a denaturant for the otherwise insoluble protein. Histidine tagged proteins were extracted from the supernatant of cell lysates using HisPur nickel spin columns (Quiagen) which utilize nickel-chelate affinity chromatography. Each protein solution underwent sequential dialysis steps against decreasing concentrations of urea using Slide-A-Lyzer dialysis cassettes (Thermo Scientific) with the final urea concentrations of 2M in Tris-HCL buffer. Each recombinant capsid protein was confirmed by Western blot using IRDye800 conjugated with antibody against poly-histidine residues, and visualized with an Odyssey Imager (LI-COR Biosciences) at 800nm. Additionally, Western blot was also performed using a hyperimmune serum from a pig immunized with genotype 1 HEV antigen as the primary detecting antibody [20]. The secondary antibody was IRDye800 conjugated with anti-swine antibody as previously described [32]. The concentration of each protein was analyzed with a spectrophotometer at a wavelength of 260 nm. Each protein was diluted with the final purification elution buffer to a concentration of 200µg/ml and stored at -80°C. Each step of the protein extraction and nickel purification was performed on ice and solutions contained urea as a denaturant as well as an EDTA-free protease inhibitor.

### **Challenge HEV virus stocks**

The genotype 3 HEV (Meng strain) virus stock with a  $10^{4.5}$  50% pig infectious dose (PID<sub>50</sub>) was used as the challenge virus. The generation of the infectious virus stock was reported previously [57, 62].

### **Vaccination and challenge experimental design**

Thirty SPF pigs that were negative for IgG anti-HEV were separated into 5 groups of 6 pigs each and housed in a BSL-2 facility. Pigs in each group were vaccinated intramuscularly with 1ml of PBS (later challenged with PBS as negative control), PBS (later challenged with HEV as positive control), swine HEV capsid antigen, rat HEV capsid antigen, or avian HEV capsid antigen. At two weeks post-vaccination, a booster dose of each antigen was administered to each pig. Each ml of vaccine contained approximately 200µg of respective antigen and was administered equally (0.5 ml) to muscles on each side of the neck. At 4 weeks post-vaccination when the vaccinated pigs developed a high titer of IgG anti-HEV, all pigs with the exception of the negative control group were intravenously challenged with 1 ml of the prototype genotype 3 HEV (Meng strain) containing  $10^{4.5}$  PID<sub>50</sub> HEV. The negative control pigs were challenged with PBS buffer. All pigs were necropsied at 4 weeks post-challenge at which time the liver histological lesions peaked [63].

### **Sample collection**

Serum samples and fecal swab materials were collected prior to vaccination and weekly thereafter from each pig. Following vaccination, the weekly serum samples were tested for IgG anti-HEV by an ELISA [20]. After challenge, the serum and 10% fecal samples were tested weekly for the presence of HEV RNA by a nested RT-PCR. Liver, bile, and small intestinal contents were collected at necropsy. Bile and 10% small intestinal contents were tested for HEV RNA by the nested RT-PCR while liver samples

were evaluated for lymphoplasmacytic infiltrates by a pathologist using a scoring system previously described [63].

### **RT-PCR to detect HEV RNA**

Serum and fecal samples were tested by a genotype 3 HEV-specific nested RT-PCR as described previously [57] except that the strain specific primers targets the ORF1 gene. Briefly, total RNAs were extracted from 100 µl of 10% fecal suspension or serum samples using TriZol reagents (Life Technologies) and resuspended in 30 µl of sterile water. Reverse transcription reactions were performed at 42°C for 1 hr with 1 µl (10 µM) of the external reverse primer (5'-CGATCTCTAACGCTAGGTTG-3'), 1 µl (200 units/µl) of Superscript II reverse transcriptase (Life Technologies), 1 µl of 0.1M dithiothreitol, 4 µl of 5x RT buffer, 0.5 µl (40 units/µl) of RNasin ribonuclease inhibitor (Promega), and 1 µl of 10 mM deoxynucleoside triphosphates. For the first round PCR, forward primer (5'-TCGTCTCCTCTACACCTATC-3') and reverse primer (5'-CGATCTCTAACGCTAGGTTG -3') were used to amplify a 471 bp product. For the second round nested PCR, forward primer (5'-CGACTGAGTTCATTATGCGTG-3') and reverse primer (5'-CCTCAGTTATAGTAAGCGCC-3') were used to amplify a 277 bp fragment using 5 µl of the first round PCR product as the template using AmpliTaq Gold DNA polymerase (Applied Biosystems). The cycling parameters included an initial denaturation/polymerase activation step at 95°C for 9 min, followed by 39 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 52°C, extension for 1 min at 72°C, and a final extension step at 72°C for 7min. The resulting PCR products were analyzed via electrophoresis on a 1% agarose gel.

### **Enzyme-linked immunoabsorbent assay (ELISA) to detect IgG anti-HEV**

Pig serum samples were tested by an ELISA as previously described [20, 57, 64] except that the respective homologous recombinant purified protein expressed from swine, avian and rat HEVs in this study was used. The ELISA cutoff was set at 3 standard deviations above the mean OD values of the pre-vaccinated samples. All sera were tested in duplicate at a 1:100 dilution.

### **Statistical analyses**

Histopathologic lesions were recorded as lesion scores. The mean lesion scores were compared by weighted least squares analysis of variance using the TTEST ANOVA function in Office Excel (Microsoft, INC. 2007).

## **RESULTS**

### **Characterization of bacteria-expressed truncated recombinant capsid antigens of swine, rat, and avian HEV**

The truncated ORF2 proteins derived from swine, avian and rat HEV were each expressed as insoluble inclusion bodies with an approximate predicted size of 58kDa before histidine tagging [65, 66]. Following nickel affinity chromatography purification, the poly-histidine tag fused to each protein was properly identified on each of the recombinant fusion proteins using Western blot with IRDy800 (LI-COR Biosciences) conjugated antibodies to poly-histidine tags (Fig.1A). Further Western blot analyses using polyclonal hyperimmune swine antiserum raised against genotype 1 human HEV confirmed the expression of swine, rat, and avian ORF2 proteins (Fig. 1B). The

antiserum raised against genotype 1 human HEV cross-reacted to the truncated recombinant capsid antigens from genotype 3 swine HEV, avian HEV and rat HEV (Fig. 1B), further confirming the existence of a single HEV serotype.

### **The truncated recombinant capsid antigens derived from swine, rat, or avian HEV induced strong antibody responses in vaccinated pigs**

Pigs vaccinated intramuscularly with swine, rat, or avian HEV capsid antigens all developed strong IgG anti-HEV antibody as detected with antigen-specific ELISA (Fig 2B, 2C, 2D). There is no detectable difference in the level and pattern of antibody responses among the vaccinated groups, indicating that capsid antigens from different sources are capable of inducing antibody production. After a booster dose at two weeks after the initial vaccination, all pigs in each group seroconverted to IgG anti-HEV. The control pigs that were vaccinated with PBS were negative for IgG anti-HEV antibody (Fig 2A).

### **Hepatic lesion scores at 56 days post vaccination (dpv)**

Sections of liver samples from each pig were collected at necropsy and processed for histological examination for the presence or absence of lymphoplasmacytic hepatitis (Table 2) by a pathologist using a previously-described scoring system [63]. Only mild lymphoplasmacytic hepatitis (score of 1) was observed (Table 2), which is consistent with previous studies [63], as genotype 3 swine HEV infection is generally subclinical in pigs [20]. In general, the pigs vaccinated with avian ( $P = .01$ ), swine ( $P = .289$ ) or rat ( $P = .289$ ) HEV antigens had lower average mean histological scores when compared to the

unvaccinated and challenged pigs (Table 2). Interestingly, the group with the lowest average hepatic lesion score was vaccinated with avian HEV ORF2 antigen.

### **Detection of HEV RNA in serum and feces with RT-PCR**

After challenge, the detection of HEV RNA was performed using a nested RT-PCR with primers that target the ORF1 gene regions. Positive and negative control serum or fecal suspension of the genotype 3 HEV was included in all steps of the RNA extraction, reverse transcription and nested PCR processes to ensure the RT-PCR works properly. After challenge, HEV viremia is not detected in any pigs, which is consistent with the nature of genotype 3 swine HEV infection in pigs (Table 3). All 6 unvaccinated pigs shed virus in feces after challenge, and the virus shedding lasted 1-2 weeks. In contrast, pigs vaccinated with swine HEV or avian HEV had only 3 pigs within each group that shed virus in feces, although the pigs vaccinated with rat HEV antigen all had fecal virus shedding. At necropsy at 4 weeks post-challenge, HEV RNA was not detected in the bile or small intestinal contents of any pig. As expected, the negative control pigs challenged with PBS buffer had no viremia or fecal virus shedding at any time during the study.

## DISCUSSION

The current experimental HEV vaccines are all based on the recombinant capsid protein derived from a single strain of HEV. Although the vaccines are very promising in human clinical trials [44, 45], their efficacy against the genetically diversified genotypes of HEV needs to be evaluated. The identification of numerous genetically distinct animal strains of HEV and the demonstrated cross-species infection by these animal strains further raise the concern as to whether or not a vaccine based on a single HEV strain will confer sufficient protection against strains from different genotypes especially those animal strains with zoonotic potential. To our knowledge, this is the first study evaluating the cross-protective efficacy of N-terminal truncated capsid antigens derived from various animal strains of HEV as candidate vaccines against HEV challenge in a pig model.

Vaccine trials evaluating the protective ability of HEV ORF2 antigens have been performed in chickens [48], non-human primates [58, 65, 67, 68], and humans [44, 45]. The results from these vaccine studies trials showed that the HEV ORF2 capsid protein induce excellent protection against HEV infection. For examples, a recombinant N-terminal truncated ORF2 capsid protein induced complete protection in rhesus monkeys against both acute hepatitis and infection after challenge with genotype 1 (Sar-55) or genotype 3 strain (US-2) HEV. Monkeys were also completely protected from hepatitis and partially protected from infection after challenge with genotype 2 (Mex-14) HEV [69], indicating effective cross-protection among the four recognized genotypes of mammalian HEV, which is desirable for a HEV vaccine as there exist at least 4 recognized and two putative genotypes of mammalian HEV worldwide [70]. In a large

scale comprehensive human vaccine trial with over 100,000 participants, 3 doses of 30µg of recombinant bacterially-expressed HEV ORF2 protein induced complete protection for at least 1 year [44], and adverse reactions to ORF2 protein vaccination were negligible. It has also been shown that previous infection with a strain of HEV likely confers protection against subsequent infections from different strains. Using rhesus macaques as a model, Huang et al. demonstrated that previous infection with genotypes 1 and 4 HEV almost completely protect the animals against challenge with heterologous or homologous strains of HEV [56]. Similar results were also found in pigs where prior infection with a genotype 3 swine HEV protected pigs against subsequent infections with genotypes 3 and 4 human HEV and partially protective against homologous genotype 3 swine HEV re-infection [57].

In this study, all 6 unvaccinated pigs had fecal virus shedding after challenge with two consecutive weeks of fecal virus shedding in half of the pigs. In contrast, in avian HEV and swine HEV antigen-vaccinated pigs, only three pigs in each group had fecal virus shedding with only 1 pig lasting 2 weeks, therefore indicating that the avian HEV and swine HEV capsid antigens induce protection against genotype 3 HEV challenge (Table 3). Although the protection is not complete in all pigs, as seen in primate and chicken HEV ORF2 vaccine studies, these results from this study do indicate a cross-protection of the avian HEV ORF2 capsid antigen against genotype 3 mammalian HEV challenge. In pigs vaccinated with the rat HEV capsid antigen, all challenged pigs had fecal virus shedding similar to the unvaccinated challenged control pigs, suggesting that the rat HEV antigen does not induce sufficient level of protection against genotype 3 HEV. This is not surprising as the rat HEV is genetically very divergent from human

HEVs, and in fact the rat HEV belongs to a unique genotype [14, 19]. It is possible that the rat HEV may not share common neutralizing epitopes in the capsid with the genotype 3 HEV. Although the avian HEV shares only 50-60% sequence identity with mammalian HEV strains [3, 4, 36], the avian HEV may be more antigenically related to the mammalian HEV. In fact, the capsid protein of avian HEV has been shown to share common antigenic epitopes with the human HEVs [71, 72].

In general, histological hepatic lesions characterized by lymphoplasmacytic inflammation is mild in genotype 3 swine HEV-infected pigs. At necropsy at 4 weeks post-challenge, the mean liver lesion score in the unvaccinated/challenged group is higher than all three vaccinated/challenged groups, suggesting a certain level of cross-protection of the three animal HEV antigens against genotype 3 HEV which further support the fecal virus shedding data. Interestingly, the pigs vaccinated with the avian HEV ORF2 antigen had no histological liver lesions, further indicating that avian HEV capsid antigen confers cross-protection against genotype 3 mammalian HEV. The inability to propagate HEV in a cell culture system to produce a higher titer challenge virus stock and the subclinical infection nature of genotype 3 HEV in pigs limited our ability to conduct a robust challenge study and fully assess the cross-protective ability of these animal HEV-derived antigens. We are also limited by the fact that serum and fecal samples were collected one day per week whereas daily samples would provide a better picture of the actual duration of fecal virus shedding. The lack of viremia in even the unvaccinated/challenged pigs is not unexpected since viremia is often absent or transient in HEV-infected pigs [32, 57, 64, 73]. Nevertheless the combined histological evaluation and fecal virus shedding data suggest that the capsid antigens derived from avian, swine

and rat HEV confer at least partial cross-protection against genotype 3 HEV challenge in pigs.

The results from the Western blot analysis revealed that the truncated capsid antigens derived from swine, rat, and avian HEV cross-reacted with polyclonal anti-HEV antiserum raised against genotype 1 human HEV [20]. Similarly, the rabbit HEV antiserum cross-reacted strongly with truncated swine and human ORF2 protein and weakly with rat and avian ORF2 protein, and conversely the rabbit HEV ORF2 antigen cross-reacted strongly with genotype 1 human and genotype 3 swine HEV antiserum and weakly with avian and rat HEV antiserum [32]. Taken together, these results demonstrated antigenic cross-reactivity, which is important, but also significant antigenic variability which may explain the partial cross-protection seen in this study

In summary, pigs vaccinated with truncated recombinant capsid antigens from three animal strains of HEV induced strong antibody response in vaccinated pigs (Figure 2), which is consistent with other studies that showed robust seroconversion to IgG anti-HEV antibodies after vaccination with HEV ORF2 antigens [46, 48, 65, 74]. However, these recombinant antigens from genetically distant animal strains of HEV appear to confer only partial cross-protection against a genotype 3 mammalian HEV. The recombinant HEV ORF2 antigens in most of these other vaccine trial studies were adjuvanted with either aluminum [46, 65, 74] or keyhole limpet hemocyanin [48], while the recombinant HEV ORF2 antigens in this study were not even though strong antibody responses were elicited at the time of challenge. Clearly, additional studies are warranted to fully evaluate if the current experimental vaccines based on a single HEV strain will confer efficient protection especially against the zoonotic animal strains of HEV.

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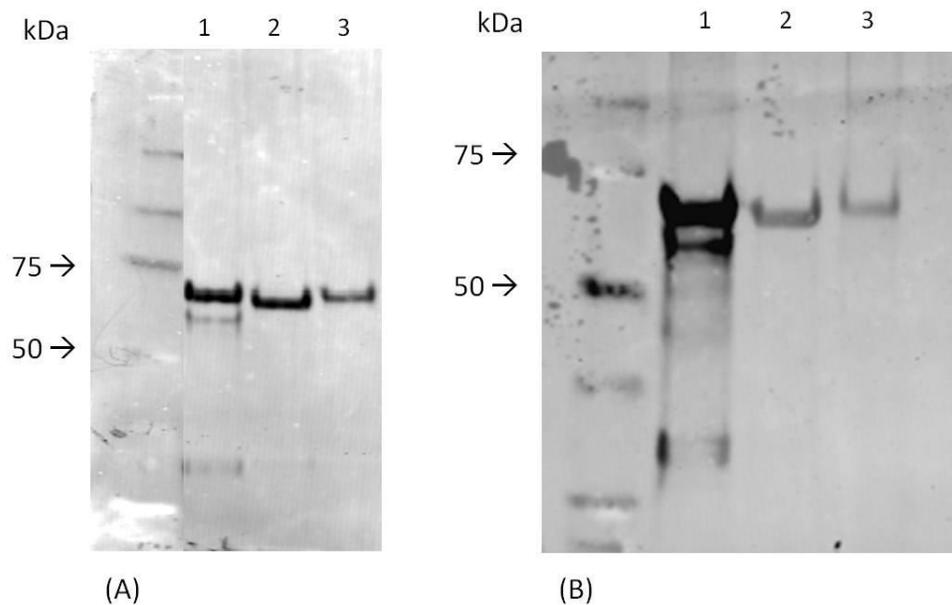
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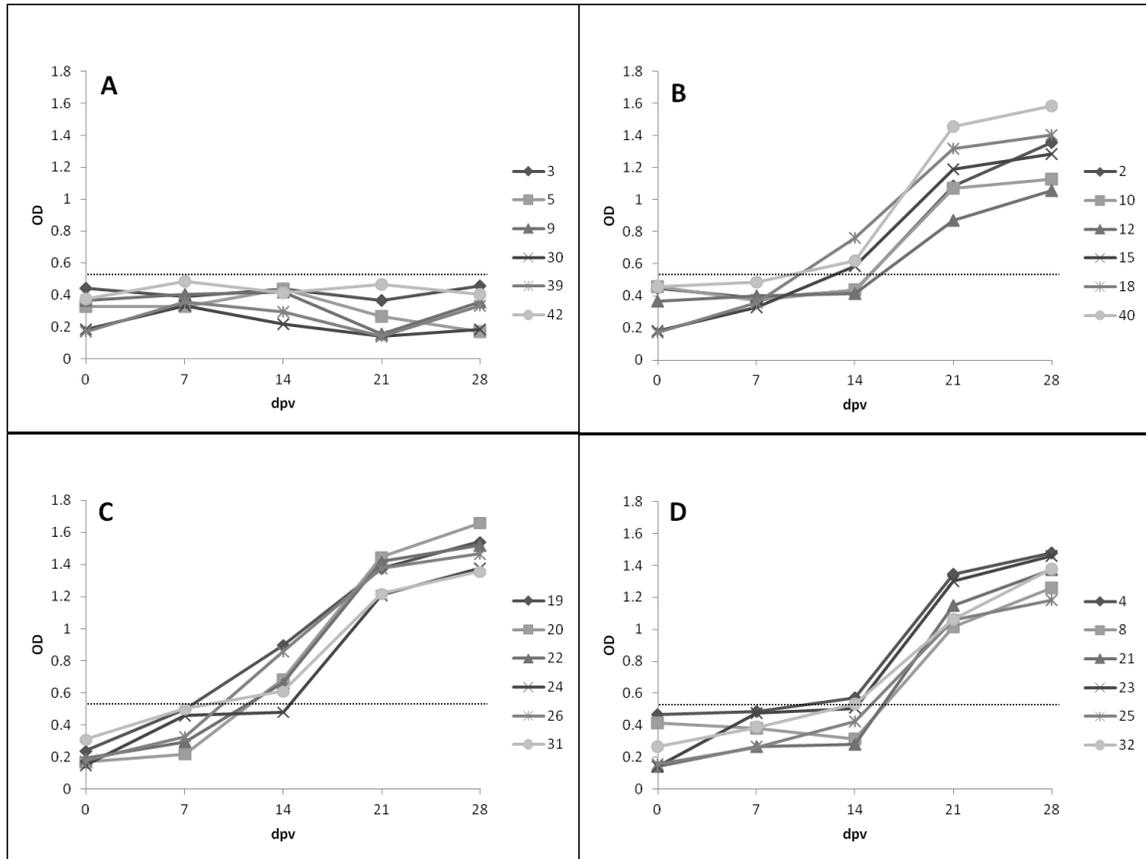
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## Figure Legends

**Figure 1.** Western blot analyses of truncated bacterially-expressed recombinant swine, rat, and avian HEV ORF2 capsid antigens. Each lane was loaded with truncated recombinant capsid proteins derived from swine HEV (1), rat HEV (2), or avian HEV (3) HEV. **Panel A:** A PVDF membrane containing separated proteins was incubated with IRDye800 conjugated antibody against 6-His residue tags to detect the respective fusion capsid proteins. **Panel B:** A PVDF membrane containing separate proteins was first incubated with a hyperimmune swine antiserum raised against the genotype 1 human HEV followed by incubation with IRDye800 conjugated antibody against swine IgG. Both membranes were read at wavelength 800nm using an Odyssey Imager (LI-COR biosciences).



**Figure 2.** IgG anti-HEV antibody responses in pigs vaccinated with PBS buffer (A), swine HEV ORF2 antigen (B), rat HEV ORF2 antigen (C), or avian HEV ORF2 antigen (D) as tested by ELISA. Pigs were vaccinated on day 0 and a booster dose was given on day 14.



**Table 1.** Oligonucleotides used in PCR for amplification of truncated ORF2 proteins

Product	Oligo Sequence
Truncated swine ORF2	5'-AAGGATCCATGGCTGTATCACCGGCCCCCGAC-3'
	5'-AACTCGAGTTAAGACTCCCGGGTTTTACC-3'
Truncated rat ORF2	5'-CATCATGGATCCGCACAGGCACCGGC-3'
	5'-CATCATAGATCTTCAGACACTATCGGCGGC-3'
Truncated avian ORF2	5'-CATCATGGATCCGACGTTGTCACCGCGG-3'
	5'-CATCATAGATCTTTAGGGTGGTGAGGGGAATG-3'

<sup>a</sup>All sera were tested at a 1:100 dilution

**Table 2.** Hepatic histological lesion scores at 4 weeks post-challenge in vaccinated and control pigs challenged with a genotype 3 HEV

Vaccine group	Pig ID#	Challenge inocula	Liver histological lesion score <sup>a</sup>	Mean histological liver lesion Score
PBS	11	PBS	0	.17
	13		1	
	14		0	
	16		0	
	17		0	
	29		0	
Swine HEV ORF2	2	Genotype 3 swine HEV	0	.33 <sup>cf</sup>
	10		0	
	12		1	
	15		0	
	18		0	
	40		1	
Rat HEV ORF2	19	Genotype 3 swine HEV	0	.33 <sup>be</sup>
	20		0	
	22		1	
	24		0	
	26		0	
	31		1	
Avian HEV ORF2	4	Genotype 3 swine HEV	0	0 <sup>def</sup>
	8		0	
	21		0	
	23		0	
	25		0	
	32		0	
PBS	3	Genotype 3 swine HEV	0	.67 <sup>bcd</sup>
	5		1	
	9		0	
	30		1	
	39		1	
	42		1	

<sup>a</sup>Lymphoplasmacytic scores 0 = no inflammation, 1=1 to 2 lymphoplasmacytic infiltrates/10 hepatic lobules, 2 = 3 to 5 focal infiltrates/10 hepatic lobules, 3 = 6 to 10 focal infiltrates/10 hepatic lobules, and 4 = >10 focal infiltrates/10 hepatic lobules (Halbur et al., 2001).

<sup>b</sup> PBS vaccine vs. rat ORF2 ( $P = .289$ )

<sup>c</sup> PBS vaccine vs. swine ORF2 ( $P = .289$ )

<sup>d</sup> PBS vaccine vs. avian ORF2 ( $P = .01$ )

<sup>e</sup> avian ORF2 vs. rat ORF2 ( $P = .144$ )

<sup>f</sup> avian ORF2 vs. swine ORF2 ( $P = .144$ )

**Table 3.** Fecal virus shedding and viremia in vaccinated and control pigs after challenge with a genotype 3 swine HEV

Vaccine antigen	Pig ID#	Challenge virus inocula	Presence (+) or absence (-) of HEV detected in serum/feces samples at indicated week post-challenge (wpc)				
			0	1	2	3	4
PBS	11	PBS	-/-	-/-	-/-	-/-	-/-
	13		-/-	-/-	-/-	-/-	-/-
	14		-/-	-/-	-/-	-/-	-/-
	16		-/-	-/-	-/-	-/-	-/-
	17		-/-	-/-	-/-	-/-	-/-
	29		-/-	-/-	-/-	-/-	-/-
Swine HEV ORF2	2	Genotype 3 swine HEV	-/-	-/-	-/-	-/-	-/-
	10		-/-	-/+	-/+	-/-	-/-
	12		-/-	-/-	-/-	-/-	-/-
	15		-/-	-/+	-/-	-/-	-/-
	18		-/-	-/+	-/-	-/-	-/-
	40		-/-	-/-	-/-	-/-	-/-
Rat HEV ORF2	19	Genotype 3 swine HEV	-/-	-/-	-/+	-/-	-/-
	20		-/-	-/+	-/-	-/-	-/-
	22		-/-	-/+	-/+	-/-	-/-
	24		-/-	-/+	-/+	-/-	-/-
	26		-/-	-/-	-/+	-/-	-/-
	31		-/-	-/-	-/+	-/-	-/-
Avian HEV ORF2	4	Genotype 3 swine HEV	-/-	-/-	-/-	-/-	-/-
	8		-/-	-/-	-/-	-/-	-/-
	21		-/-	-/-	-/-	-/-	-/-
	23		-/-	-/+	-/-	-/-	-/-
	25		-/-	-/+	-/-	-/-	-/-
	32		-/-	-/+	-/+	-/-	-/-
PBS	3	Genotype 3 swine HEV	-/-	-/-	-/+	-/-	-/-
	5		-/-	-/-	-/+	-/-	-/-
	9		-/-	-/+	-/+	-/-	-/-
	30		-/-	-/+	-/+	-/-	-/-
	39		-/-	-/+	-/+	-/-	-/-
	42		-/-	-/+	-/-	-/-	-/-

## CHAPTER 4

### Serological evidence for a hepatitis E virus (HEV)-related agent in goats in the United States

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#### ABSTRACT

Hepatitis E virus (HEV) causes an important public health disease in many developing countries and is also endemic in some industrialized countries. In addition to humans, strains of HEV have been genetically identified from pig, chicken, rat, mongoose, deer, rabbit and fish. While the genotypes 1 and 2 HEV are restricted to humans, the genotypes 3 and 4 HEV are zoonotic and infect humans and other animal species. As a part of our ongoing efforts to search for potential animal reservoirs for HEV, we tested goats from Virginia for evidence of HEV infection and showed that 16% (13/80) of goat sera from Virginia herds were positive for IgG anti-HEV. Importantly, we demonstrated that neutralizing antibodies to HEV were present in selected goat sera with higher titers of IgG anti-HEV. Subsequently, in an attempt to genetically identify the HEV-related agent from goats, we conducted a prospective study in a closed goat herd with known anti-HEV seropositivity and monitored a total of 11 kids from the time of birth until 14 weeks of age for evidence of HEV infection. Seroconversion to IgG anti-

HEV was detected in 7 out of the 11 kids, although repeated attempts to detect HEV RNA by a broad-spectrum nested RT-PCR from the fecal and serum samples of the goats that had seroconverted were unsuccessful. In addition, we also attempted to experimentally infect laboratory goats with three well-characterized mammalian strains of HEV but with no success. The results indicate that a HEV-related agent is circulating and maintained in the goat population in Virginia and that the goat HEV is likely genetically very divergent from the known HEV strains.

## INTRODUCTION

Hepatitis E virus (HEV) is an important cause of acute viral hepatitis throughout the world (Emerson and Purcell, 2003). Once believed to cause diseases in only developing countries, HEV is now recognized as the causative agent of sporadic cases of acute hepatitis E in many industrialized countries such as the United States, Japan, and many European countries (Emerson and Purcell, 2003, Meng, 2010a, Meng, 2010b, Yazaki et al., 2003, Galiana et al., 2008, Brost et al., 2010, Colson et al., 2010, Dalton et al., 2008, Dalton et al., 2007). HEV is transmitted via the feco-oral route, and causes large waterborne outbreaks and sporadic disease but is not thought to transmit easily from human to human (Purdy and Khudyakov, 2011). HEV is a single-stranded, positive-sense, non-enveloped RNA virus that is classified in the family *Hepeviridae* with at least 4 major genotypes (Emerson and Purcell, 2003, Meng, 2011). Genotypes 1 and 2 are restricted to humans and are mainly associated with epidemics in developing countries, whereas genotypes 3 and 4 HEV strains are zoonotic and associated with sporadic cases of hepatitis E with a worldwide distribution in both humans and other animal species.

The overall mortality of HEV infection is <1% and it is a leading cause of acute viral hepatitis worldwide (Emerson and Purcell, 2003). The mortality rate associated with HEV infection increases up to 28% in infected pregnant women (Emerson and Purcell, 2003). Recently, neurological manifestations in some HEV-infected patients have been reported (Kamar et al., 2011, Aggarwal, 2011, Despierres et al., 2011), although the mechanism of action is unclear. The disease is usually acute and self-limiting, although chronic infections have recently been reported in immunosuppressed individuals such as HIV/AIDS patients (Dalton et al., 2009, Kenfak-Foguena et al., 2011, Kaba et al., 2011,

Keane et al., 2012) and organ transplant recipients (Aggarwal, 2008, Kamar et al., 2008, Pischke et al., 2010, Legrand-Abravanel et al., 2011). Although only sporadic or cluster cases of hepatitis E have been reported in individuals from industrialized countries, seroepidemiological studies revealed a surprisingly high prevalence of IgG anti-HEV in individuals from industrialized countries: approximately 20% in the United States (Kuniholm et al., 2009) and up to 52% in Southern France, thus suggesting an unknown source of exposure (Mansuy et al., 2011).

In addition to humans, strains of HEV have also been genetically identified from a number of other animal species including domestic and wild pigs (de Deus et al., 2008), deer (Tei et al., 2003), rabbits (Cossaboom et al., 2011, Zhao et al., 2009), chickens (Payne et al., 1999), rats (Johne et al., 2010a, Purcell et al., 2011), and even trout (Batts et al., 2011). To date, the only definitive transmissions of HEV from animals to humans resulted from consumption of infected animal meats (Colson et al., 2010, Yazaki et al., 2003, Takahashi et al., 2004, Tei et al., 2003). Therefore, it seems reasonable to speculate that any other major zoonotic reservoir for human hepatitis E might be an animal common in the human food chain. Because goat meat is consumed in many countries and anti-HEV antibodies have been reported in goats (Arankalle et al., 2001, Peralta et al., 2009), in this study we explored the possibility that goats might be a reservoir for human HEV infections.

## MATERIALS & METHODS

**Goat serum samples:** A total of 50 serum samples of mature goats including 49 female and 1 male were collected in 2002 from Virginia (Table 1). In addition, we also collected serum samples of 30 additional goats from two separate goat herds in Southwest Virginia. Both herds are predominantly closed with a very limited number of new animals entering each year. Herd A is a purebred herd of Myotonic Goats, and herd B is genetically diverse with cross-bred animals (Table 1).

**Sources of viruses:** Three well-characterized infectious stocks of genotypes 1 human HEV (strain Sar-55) (Tsarev et al., 1994), genotype 3 swine HEV (Meng strain) (Meng et al., 1997, Meng et al., 1998b), and genotype 4 human HEV (strain TW6196E) (Feagins et al., 2008) were used in the experimental goat transmission study.

**Experimental inoculation of laboratory goats with HEV:** Twelve anti-HEV negative goats with diverse age and genetic background were obtained from a commercial herd through the Virginia Tech Lab Animal Resources. The animals were divided into 4 groups of 3 goats each, and were housed in separate rooms in a BSL-2 animal facility at Virginia Polytechnic Institute and State University. Goats in group 1 served as negative control and were intravenously (IV) administered 1 ml PBS buffer. The three goats each in groups 2, 3 and 4 were inoculated IV with  $2 \times 10^{4.5}$  50% monkey infectious dose (MID<sub>50</sub>) of a genotype 3 HEV, or with  $2 \times 10^3$  MID<sub>50</sub> of a genotype 4 human HEV or a genotype 1 human HEV, respectively. Serum and fecal samples were collected from each animal prior to inoculation and weekly thereafter for a total of 9 weeks post-

inoculation, and serum samples were tested by ELISA for seroconversion to IgG anti-HEV. All animals were humanely euthanized at the end of the 9-week study.

**Prospective field study to identify HEV from goats:** In an attempt to identify the HEV-related agent from goats, we performed a prospective field study in a closed goat herd that is known to be seropositive for HEV antibodies. Briefly, a total of 11 young goats were selected from IgG anti-HEV negative dams in the herd, tagged and monitored for evidence of HEV infection from the time of birth until 14 weeks of age. The 11 study goats from the herd were allowed to freely mingle with the other goats in the same herd. Weekly serum and fecal samples were collected from each of the 11 goats and tested for the presence of HEV RNA (see below), and the weekly serum samples were also tested by an ELISA for seroconversion to IgG anti-HEV.

**RT-PCR to detect HEV from goat samples:** Selected serum and fecal samples were tested by a broad-spectrum RT-PCR for the presence of HEV RNA essentially as described by Johne et al (8). Degenerate nested-PCR primer pairs were designed on the basis of a multiple sequence alignment of known HEV strains. Following Trizol (Life Technologies) extraction, total RNAs were extracted from 150  $\mu$ l of 10% fecal suspension or serum samples, and resuspended in 30  $\mu$ l of sterile water. Reverse transcription reactions were performed at 42°C for 1 hr with 1  $\mu$ l (10  $\mu$ M) of the external reverse degenerate primer (5'-GCCATGTTCAGACDGTRTTCCA-3'), 1  $\mu$ l (200 units/ $\mu$ l) of Superscript II reverse transcriptase (Life Technologies), 1  $\mu$ l of 0.1M dithiothreitol, 4  $\mu$ l of 5x RT buffer, 0.5  $\mu$ l (40 units/ $\mu$ l) of RNasin ribonuclease inhibitor

(Promega), and 1  $\mu$ l of 10 mM deoxynucleoside triphosphates. For the first round PCR, forward primer (5'-TCGCGCATCACMTTYTTCCARAA-3') and reverse primer (5'-GCCATGTTTCAGACDGTRTTCCA-3') were used to amplify a 470 bp product. For the second round nested PCR, forward primer (5'-TGTGCTCTGTTTGGCCCNTGGTTYMG-3') and reverse primer (5'-CCAGGCTCACCRGARTGYTTCTTCCA-3') were used to amplify a 330 bp fragment using 5  $\mu$ l of the first round PCR product as the template using AmpliTaq Gold DNA polymerase (Applied Biosystems). The cycling parameters included an initial denaturation/polymerase activation step at 95°C for 9 min, followed by 39 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 42-52°C, extension for 1 min at 72°C, and a final extension step at 72°C for 7min. The resulting PCR products were analyzed via electrophoresis on a 1% agarose gel with ethidium bromide.

In addition, we also utilized another RT-PCR protocol for the first round PCR amplification using a Qiagen one-step RT-PCR kit (Qiagen Inc.) combined with a second round PCR amplification with a TaKaRa ExTaq kit (TaKaRa Bio. Inc.). The PCR parameters using the Qiagen one-step kit were 42°C for 60 min of the reverse transcription reaction, 95°C for 15 min of denaturation/polymerase activation followed by 40 cycles of 94°C for 30 sec of denaturation, 50 °C for 30 sec annealing, and 74°C for 45 sec of extension, with a final extension at 74°C for 5 min. The PCR parameters for the second round TaKaRa PCR included a denaturation/polymerase activation step at 95°C for 5 min followed by 35 cycles of denaturing at 30 sec at 94°C, annealing for 30 sec at 50°C, extension for 45 sec at 72°C, and a final extension step at 72°C for 5 min (Johne et al., 2010b).

**Enzyme-linked immunoabsorbent assay (ELISA) to detect IgG anti-HEV in goats:**

The goat serum samples were tested for IgG anti-HEV by an ELISA with the genotype 1 human HEV recombinant capsid antigen essentially as described previously (Engle et al., 2002). All sera were tested at a 1:100 dilution. A commercial goat serum sample from a supplier that contained a high titer of IgG anti-HEV was used as a positive control for the ELISA.

***In vitro* neutralization test:** An *in vitro* neutralization test for HEV was performed essentially as described previously (Emerson et al., 2006) on selected goat sera to determine if anti-HEV neutralizing antibodies might be present in goat serum samples with higher titers of IgG anti-HEV (Table 2). All sera were tested at 1:100 dilution. Briefly, triplicate samples of human HEV genotype 1 (strain Sar55) were incubated with PBS or selected goat sera (Table 2), and plated under code on human hepatocellular carcinoma cells HepG2/C3A. Six days later, cells were stained by an immunofluorescence assay (IFA) with an antibody specific to HEV capsid protein (chimp 1313) and the stained cells were then counted for positive cells with IFA signals.

## RESULTS

**The prevalence of IgG anti-HEV in goats varied from herd to herd in Virginia:** We serendipitously found that a commercial goat serum sample from one supplier, but not from another, contained a high titer of IgG anti-HEV when tested by an ELISA with the genotype 1 HEV recombinant capsid antigen. Therefore, this commercial goat serum was used as a positive control in the ELISA. Among the 50 archived goat serum samples tested in this study, we found that 4% (2/50) of them were seropositive (Table 1). Subsequently, we sampled additional goats from two separate closed herds in Southwest Virginia. We found that 9 of the 9 goats sampled in herd A, and 2 of the 21 goats sampled in herd B were positive for IgG anti-HEV (Table 1).

**Presence of anti-HEV neutralizing antibodies in selected goat sera:** The *in vitro* neutralization test results showed that neutralizing antibodies were present in selected goat sera with higher IgG anti-HEV titers (Table 2). The % decreases of HEV infectivity of the selected goat sera on HepG2/C3A cells relative to PBS control were calculated. The PBS control had 208, 263, and 202 stained ORF2 IFA-positive cells (Table 2). Insufficient amounts of sera were available for further testing in some samples.

**Laboratory goats are not susceptible to experimental HEV infection:** The presence of IgG anti-HEV and neutralizing antibodies in the goat sera prompted us to conduct an experimental infection study to determine if goats could be experimentally infected with three well-characterized strains of mammalian HEV. The results showed, however, that none of the inoculated goats exhibited any clinical signs consistent with acute hepatitis at

any time during the study. Seroconversion to IgG anti-HEV was not detected in any of the inoculated or control animals during the 9-week experiment, indicating that goats are not susceptible to experimental infection by these three well-characterized genotypes of HEV with known infectious titers.

**Serological evidence of a HEV-related agent circulating in the goat herd:** In an attempt to genetically identify the HEV-related agent and to characterize the course of HEV infection in goats under field conditions, we conducted a prospective study in a goat herd located in southwest Virginia. The results showed that, during this 14-week study, 7 of the 11 young goats had seroconverted to IgG anti-HEV at some points during the course of the study (Figure 1). The overall pattern of seroconversion to IgG anti-HEV appears to be similar to natural HEV infection in young pigs where a large proportion of the animals are infected within the first few months of life (Feng et al., 2011). Among the seropositive goats, the kid #92 had a very high level of IgG anti-HEV starting at approximately 7 weeks of age and lasting until the end of the study (Figure 1). At no time did any goats exhibit clinical signs consistent with hepatitis.

**Failure to genetically identify HEV from the fecal or serum samples of the goats with seroconversions:** A broad-spectrum nested RT-PCR was used to test the goat serum and fecal samples collected from the goats with seroconversions, especially for those samples collected 2-3 weeks before, the week of, and the week after seroconversion to IgG anti-HEV. Unfortunately, no HEV-specific sequence could be amplified from these samples collected from the prospective study despite repeated RT-PCR testing.

Beside using the broad-spectrum RT-PCR assay, we also attempted to amplify HEV-specific sequence from the samples with different RT-PCR protocols and reagents but the result was the same.

## **DISCUSSION**

The first animal strain of HEV, swine hepatitis E virus (swine HEV), was identified and characterized from pigs in the United States in 1997 (Meng et al., 1997). Since then, swine HEV has been detected in pigs from essentially all swine-producing countries, and all swine HEV strains identified to date belong to either genotype 3 or genotype 4. However, recently a unique strain of HEV that may represent a new genotype was identified from wild boars in Japan (Takahashi et al., 2011). Experimental cross-species infections have demonstrated that genotypes 3 and 4 strains of swine HEV infect non-human primates, and conversely, genotypes 3 and 4 human HEV strains infect pigs (Feagins et al., 2008, Meng et al., 1998b, Arankalle et al., 2006, Meng, 2010a, Meng, 2010b). It has also been shown that pig handlers such as swine veterinarians and pig farmers are at increased risk of zoonotic HEV infection (Drobeniuc et al., 2001, Meng et al., 2002). Commercial pig livers sold in local grocery stores in many countries such as the United States, the Netherlands, and Japan are contaminated by HEV (Feagins et al., 2007, Yazaki et al., 2003), and most importantly the contaminating virus remains infectious in the pork product (Feagins et al., 2007). In fact, food-borne HEV transmissions have been definitively linked to the consumption of undercooked or raw pork products (Matsuda et al., 2003, Colson et al., 2010). It is now recognized that genotypes 3 and 4 of HEV are zoonotic viruses, and pigs are reservoirs for HEV.

Strains of HEV have also been genetically identified from a number of other animal species (Meng, 2010a, Meng, 2010b). Avian hepatitis E virus (avian HEV) isolated from chickens also has the ability to cross species barriers and infect turkeys under experimental conditions (Sun et al., 2004). However, the avian HEV is unlikely to infect humans since it failed to infect rhesus monkeys, a HEV-susceptible surrogate of man (Huang et al., 2004). A genotype 3 HEV was identified from sika deer in Japan, and cases of acute hepatitis E have been linked to the consumption of HEV-contaminated deer meat (Tei et al., 2003). A novel strain of HEV that is a distant member of genotype 3 was isolated from farm rabbits in China (Zhao et al., 2009) and the United States (Cossaboom et al., 2011), and more recently a novel rat strain of HEV was identified in Germany (Johne et al., 2010b) and the United States (Purcell et al., 2011). Both the Chinese and U.S. rabbit HEV strains were recently shown to have the ability to cross species barriers and infect pigs (Cossaboom, 2012).

In addition to pig, chicken, deer, rat, mongoose and rabbit (Meng et al., 1997, Haqshenas et al., 2001, Tei et al., 2003, Johne et al., 2010b, Nakamura et al., 2006, Cossaboom et al., 2011) from which HEV strains have been definitively identified and characterized, IgG anti-HEV has been detected in numerous other animal species including dogs, cats, cattle, horses, rodents, and goats (Liu et al., 2009, Okamoto et al., 2004, Zhang et al., 2008, Geng et al., 2011, Hirano et al., 2003, Arankalle et al., 2001, Peralta et al., 2009). However, the sources of seropositivity in these species remain largely unknown. It is important to determine if these animal species harbor a virus closely-related to the known strains of HEV infecting humans and thus serve as reservoir

or if these species contain genetically very divergent strains of HEV such as the avian HEV.

As a part of our ongoing efforts to search for potential animal reservoirs for HEV, in this study we report that goats in the United States are infected with a HEV-related agent. For the first time, we documented the presence of IgG anti-HEV in goats in the United States. Most importantly, we demonstrated that, under field conditions, goats in a closed herd from Virginia are naturally infected by a HEV-related agent as evidenced by seroconversion to IgG anti-HEV in kids from the prospective study. This is consistent with serological studies from India (Arankalle et al., 2001), China (Wang et al., 2002), and Spain (Peralta et al., 2009) that also report the presence of IgG anti-HEV antibody in goats. The demonstration of the presence of potential neutralizing antibodies in seropositive goats suggested that the agent infecting goats is antigenically and genetically related to human HEV.

Usmanov et al. (1994) reportedly infected sheep with two human HEV isolates and the infected sheep developed clinical signs consistent with viral hepatic infection (Usmanov et al., 1994). However, in this study we were unable to experimentally infect goats with three well-characterized strains of mammalian HEV, suggesting that goats are likely not reservoirs for human HEV infection. However, the fact that newborn kids seroconverted to IgG anti-HEV in the prospective study indicated that there indeed exists a HEV-related agent that is circulating and maintained in the goat population in Virginia. Unfortunately, our attempts to genetically identify the HEV-related agent from goats with universal degenerate HEV primers were unsuccessful, suggesting that the putative caprine HEV infecting goats may be genetically very divergent from the known strains of

HEV. This is not surprising, since the avian HEV identified in chickens is genetically divergent from the known mammalian HEV strains with only approximately 50% nucleotide sequence identity (Huang et al., 2004), and chickens are not susceptible to experimental infections by genotypes 1, 3 or 4 strains of HEV under laboratory conditions (Sun and Meng, unpublished data). Similarly, the strain of HEV recently identified from cutthroat trout fish in the United States shares less than 27% sequence identity with the human HEV (Batts et al., 2011). It is likely that the goat strain of HEV has an extremely low percentage of sequence identity with the known mammalian HEV, and thus the broad-spectrum PCR based on degenerate primers is unable to amplify the virus.

In summary, we documented for the first time convincing serological evidence of HEV infection in goat population in the United States as evidenced by the presence of IgG anti-HEV antibodies and neutralizing antibodies in goats, and by the detection of seroconversion to IgG anti-HEV in young goats in a prospective study in Virginia. Our failure to experimentally transmit human or swine HEV to goats under laboratory conditions and our inability to genetically identify HEV from goats using the universal degenerate PCR primers based on the sequences of known HEV strains suggested that the virus infecting goats is genetically unique. It is imperative now to genetically characterize the putative HEV-related agent in goats as we continue to search for other potential animal reservoirs of HEV. Emerging technologies such as metagenomics and deep sequencing may hold the key to eventually identify the virus from the goats in the future.

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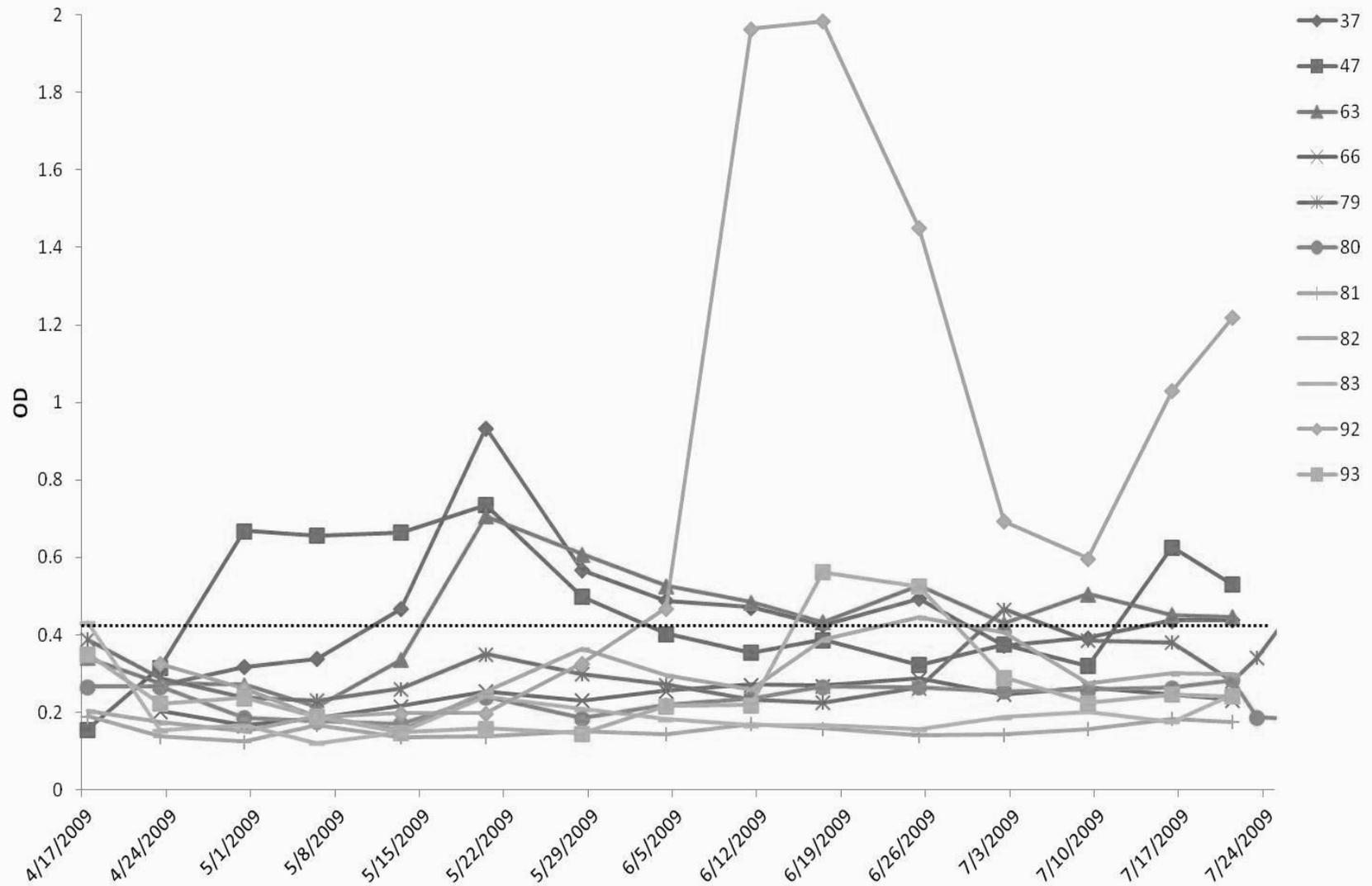
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## Figure legend

**Figure 1.** Seroconversion to IgG anti-HEV antibodies in goats from a prospective study in a closed herd in Virginia. The kids born to seronegative dams from a known seropositive goat herd were monitored for evidence of HEV infection for a total of 14 weeks from the time of birth. The weekly serum samples were tested by an ELISA for IgG anti-HEV. The ELISA OD values (Y-axis) are plotted along the X-axis which showed the ages of the animals (dates when the samples were collected).



**Table 1.** Detection of IgG HEV antibodies in sera of goats from Southwest Virginia

Herd	Year samples collected	Number of goats sampled	Number of goats positive for IgG anti-HEV (%) <sup>a</sup>
Mixed herds	2002	50	2 (4)
Herd A	2008	9	9 (100)
Herd B	2008	21	2 (9.5)

<sup>a</sup>All sera were tested at a 1:100 dilution

**Table 2.** Effect of selected goat sera on HEV infectivity in HepG2/C3A cells

Goat serum ID	IgG anti-HEV ELISA OD Value <sup>a</sup>	% Decrease in HEV infectivity relative to PBS control <sup>b</sup>
PBS	0.070	0
Commercial	1.165	ND <sup>c</sup>
G17	0.424	3%
G28	0.237	11%
G34	0.229	14%
G19	0.420	21%
G26	0.954	34%
G48	0.408	41%

<sup>a</sup> All sera were tested at 1:100 dilution

<sup>b</sup> The PBS control had 208, 263, and 202 stained cells.

<sup>c</sup> nd: not done