Pathogenesis and transmission of hepatitis E virus (HEV) in pigs

by

Chaiyan Kasorndorkbua

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Program of Study Committee:
Patrick G. Halbur, Major Professor
Mark R. Ackermann
Steven D. Sorden
Eileen J. Thacker
James A. Roth

Iowa State University

Ames, Iowa

2004

Graduate College
Iowa State University

This is to certify that the doctoral dissertation of

Chaiyan Kasorndorkbua

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

Dedicated to my mothers, Somboon Hirunsit and Siriwatana Kasorndorkbua,

and my teachers

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ABSTRACT

Human hepatitis E virus (HEV) was first reported in 1980 and is now considered a major cause of acute non-A, non-B hepatitis in humans. HEV infection is reported throughout the world and occurs in epidemic form in developing countries where the disease is endemic and is often associated with water contamination after heavy rains or flooding. Sporadic human HEV infections occur in industrialized countries where infected individuals contract the infection while traveling to endemic regions. The fecal-oral route is considered the primary mode of HEV transmission in humans. HEV infection of pigs was discovered in 1997. Since 1997, sporadic HEV infections in industrialized countries have been reported in people who have not traveled abroad and are associated with HEV isolates genetically homologous to those found in domestic pigs. HEV infection of chickens and rats has since been documented in the U.S. and abroad.

Evidence suggests that pigs serve as an important reservoir for HEV and thus exposure to pigs, pork products, or pig organs may pose a risk of zoonotic or xenozoonotic infection. Swine HEV infection causes a subclinical, non-icteric hepatitis in growing pigs. Pigs experimentally-infected with swine HEV had no signs of clinical illness; however, they were viremic for 1 to 2 weeks, HEV was present in the liver of infected pigs, and the pigs shed a large amount of HEV in feces for several weeks. Similar results were demonstrated when pigs were infected with human HEV. We demonstrated that HEV infection of pregnant pigs does not induce reproductive failure. We also demonstrated that HEV could be transmitted by intravenous exposure of pigs to feces or liver collected from pigs in the early stages of infection. We subsequently demonstrated that a high dose and/or repeated exposure may be required for fecal-oral transmission of HEV and this is likely to be a common

scenario in modern and traditional pig production facilities. HEV also can be detected in pig manure storage facilities such as concrete pits and earthen lagoons and we demonstrated that HEV found in the pit manure is viable and infectious to pigs. We attempted, but failed, to detect HEV in on-site drinking water or surface water on or near pig farms. These findings suggest a potential risk of contamination of water supplies by HEV in pig manure exists but evidence of this is lacking to date.

Cross-species infection with HEV among different species of animals has been demonstrated; swine HEV infects nonhuman primates, human HEV infects pigs, and chicken HEV infects turkeys. Swine and avian HEV have been shown to be genetically distant with nucleotide homology of approximately 60%. We demonstrated experimental infection of pigs with avian HEV. The avian HEV was found in the liver of inoculated pigs and was shed in feces for at least 3 weeks. Rat HEV failed to replicate in pigs. These findings further support the growing concern that pigs are an important reservoir of HEV and emphasize the critical role of pigs in the epidemiology of HEV.

CHAPTER 1.

GENERAL INTRODUCTION

Dissertation Organization

This dissertation is prepared as an alternate manuscript format. The dissertation includes a general introduction with a literature review, 5 separate scientific manuscripts, and general conclusions. References to literature cited in the general introduction and the general conclusions chapters are listed at the end of the dissertation. Three manuscripts have been published in refereed scientific journals relevant to the focus of the dissertation. One manuscript has recently been submitted to an appropriate journal. The Ph.D. candidate, Chaiyan Kasorndorkbua is the primary author of the manuscripts and is the principal investigator for the experimental work described in the manuscripts. The first manuscript describes the use of a swine bioassay and an RT-PCR assay to assess the risk of transmission of swine hepatitis E virus in pigs and pork products and has been published in the *Journal of Virological Methods*. The second manuscript describes the experimental infection of pregnant gilts with swine hepatitis E virus and has been published in the *Canadian Journal of Veterinary Research*. The third manuscript describes the routes of transmission of swine hepatitis E virus in pigs and has been accepted for publication in the *Journal of Clinical Microbiology*. The fourth manuscript describes the presence and infectivity of hepatitis E virus in pig manure storage facilities and on-site drinking water and nearby surface water and has been submitted to *Applied and Environmental Microbiology*. The fifth manuscript describes experimental evidence for cross-species infection of pigs with avian hepatitis E virus. This manuscript will be submitted to the *Journal of Clinical Microbiology*.

Introduction

Hepatitis E is an acute hepatitis of humans and is a major public health concern worldwide (3, 57, 69, 76). Transmission of HEV is believed to be by the fecal-oral route (19). Even though the mortality is generally low and the clinical course of hepatitis E is typically self limiting and without a chronic progressive outcome, pregnant women are prone to fatal hepatitis E with mortality rates of up to 25% (2, 19, 45, 48, 49). Hepatitis E epidemics remain a common public health problem in developing countries in parts of Africa, central Asia, and South-east Asia where HEV is endemic and individuals typically contract the infection via contaminated drinking water (3, 16, 76). Hepatitis E-associated disease has been infrequently reported in industrialized countries due to effective sanitation measures (8). However, HEV infection in humans living in non-endemic regions has recently and increasingly been documented (22, 41, 66, 67, 72, 73, 94, 97). Seroepidemiological surveys and genetic analysis of various HEV isolates indicate that there may be animal reservoirs such as pigs that serve as an important source for human HEV infections (6, 7, 33, 42, 81, 84, 93, 94, 99).

Animal strains of HEV have been identified in recent years. Swine HEV was the first known animal HEV strain and was discovered in 1997 (61). Avian HEV in chickens was identified in 2001 (30). Both swine and avian strains of HEV have been well characterized (27, 28, 29, 38). There is evidence of HEV in rats; however, rat HEV remains to be further characterized (20, 31, 33, 42). The prototype swine HEV was first discovered in the United States (U.S.) and is genetically closely related to the human strains of HEV found in the U.S. (56). Subsequently, other swine strains of HEV have been discovered globally and have been found to be ubiquitous in the pig population (7, 23, 33, 37, 57, 58, 59, 61, 86, 88, 94,

96). Human and swine HEV that are present in the same countries or geographical regions tend to be closely related genetically (36, 61, 62, 66, 81, 91). Based on genomic comparisons, the prototype swine HEV is believed to be the ancestral origin of human HEV strains found in the U.S. (29) These findings raise concern over the potential risks of pig-to-human transmission. The concern is strengthened by the findings that individuals who work with swine (i.e. swine veterinarians, pig farmers, or caretakers) are more likely to have anti-HEV antibodies than non-exposed individuals (17, 56, 58, 96). Furthermore, it has been demonstrated that swine HEV infects nonhuman primates and human HEV infects pigs experimentally (27, 59, 60).

Pork is presently a major source of protein for humans. Increasing evidence suggests that hepatitis E may also be a food-borne zoonotic disease (54, 83, 100). Consumption of raw wild boar liver or deer meat has reportedly transmitted HEV to humans (54, 83). Organs or tissues collected from swine are also used as xenografts for human patients (102).

Therefore, the first study was designed to assess the risk of transmission of swine HEV in pigs by using a swine bioassay and a RT-PCR assay. This experiment used pigs as a model to determine if there is a potential risk to transmit swine HEV via liver or pancreas as potential xenograft subjects or through pork as a food source. It remains unclear why pregnant women with HEV infection frequently develop fulminant hepatitis E with a high mortality rate. Pregnant nonhuman primates have been experimentally used as a model to investigate the pathophysiology of fulminant HEV infection in pregnant women; however, clear answers were not gained from the experiments since characteristics of HEV infection of pregnant women were not reproduced in the primate model. Therefore, in the second study, a pig model was set up to determine if experimental infection of pregnant gilts with swine

HEV may be a suitable model to study the effects of HEV infection during pregnancy and to determine if HEV is an important reproductive pathogen in pigs.

HEV infection induces subclinical hepatitis in growing pigs which exhibit no overt clinical signs (27). The natural route (s) of transmission of HEV in pigs is unknown. Since pigs may be a source of zoonotic HEV infection in humans, understanding the transmission routes of HEV in pigs is needed. Thus, the third study was initiated to investigate possible routes of HEV transmission in pigs. Pig manure is stored in large amounts on pig farms and is also commonly used as a fertilizer in agricultural practices. Contamination of the environment by pig manure is a concern in pig production areas. The fourth study was designed to investigate the presence of swine HEV in pig manure storage facilities and nearby water sources. A swine bioassay was also included in the study to determine if swine HEV found in pig manure or environmental water remained viable and infectious.

Hepatitis E virus is genetically heterogeneic and geographical clusters of HEV strains have been demonstrated (40, 71). HEV discovered in chickens (avian HEV) is genetically distant from swine HEV with approximately 60% nucleotide identity compared to over 90% identity between the U.S. swine and human HEV (38). It has been shown that avian HEV does not cross species barriers to infect nonhuman primates; however, the HEV from chickens is still able to infect and replicate in turkeys (38, 78). In some parts of the world, pigs and chickens are raised in close proximity. It is important to better understand if cross-species transmission occurs between pigs and chickens and if the virus changes with passage in another species. Serologic evidence also indicates the existence of HEV infection in rats (21, 33, 41). Rats are a potential reservoir for HEV and may also serve as an important source for human HEV infection (31). Thus, the fifth study was designed to explore the

epidemiology and comparative pathogenesis of HEV infection of pigs with viruses from pigs, chickens, and rats.

CHAPTER 2.

LITERATURE REVIEW

History

Historically, hepatitis E was associated with what are now thought to be enterically-transmitted non-A, non-B hepatitis epidemics (69). Hepatitis E associated epidemics were reported in the 1950's in India and central Asia (8). The disease was described as a distinct viral hepatitis in 1983 following experimental fecal-oral transmission of hepatitis to a human who volunteered to orally ingest feces collected from patients with hepatitis A-like disease. The volunteer developed severe clinical hepatitis and virus-like particles were found in the stool collected from the volunteer (5). Since the early 1980's, epidemics of hepatitis E have been predominantly reported in developing countries in parts of Africa, central America, and Asia. In these areas, HEV-associated disease is thought to be associated with drinking water contamination after heavy rains or flooding (16, 47). Hepatitis E has become endemic in developing countries where public hygiene is inadequately practiced (8, 75). HEV infection is rare in the industrialized countries (8, 20). However, sporadic cases of human HEV infection are reported in individuals who had a history of traveling to endemic regions (46, 68).

Viral Hepatitides in Humans

Viral hepatitis in humans can be due to infection with a variety of different viruses that cause hepatitis and jaundice due to direct or indirect injury to the liver. The human hepatitis viral agents comprise five distinct viruses classified in different virus families.

Letters from A to E are used to designate each virus based on a chronological manner of the discovery of the viruses.

Hepatitis A virus (HAV) is a major causative agent of acute hepatitis in humans. HAV infection is thought to be documented in the Middle Ages during the 8th century AD, continued to be a primary infectious problem in the armies during World War II, and remains a global health issue (34). In the past, hepatitis A was called "campaign jaundice" or "infectious hepatitis". The disease induced by HAV is commonly associated with widespread epidemics and is transmitted through the fecal-oral route. HAV is a linear, positive-strand RNA and is classified in the family *Picornaviridae*. The clinical course of HAV infection is variable and mild-to-severe in severity. Human patients with acute hepatitis A infection typically manifest jaundice due to multifocal inflammatory reactions in the affected hepatic parenchyma. Hepatocytes are primarily the site of replication. Young children are prone to inapparent HEV infection. Severe clinical hepatitis is typically found in a majority of HAV infections in adults. A mortality rate of individuals with clinical hepatitis A is low and the infection rarely induces chronic hepatitis. Inactivated or attenuated live HAV vaccines are available.

Hepatitis B virus (HBV) was previously known as "serum hepatitis" since HBV epidemics were usually associated with the use of lymph or serum for vaccination and transfusion, respectively (35). HBV is a circular, enveloped DNA virus and is classified as a *hepadnavirus* in the family *Hepadnaviridae*. HBV is a blood-borne agent and the transmission is carried out by exposure to blood via blood transfusion, unprotected sexual intercourse, parenteral injection and mother-to-infant vertical transmission. The clinical course of HBV infection is variable, depending on a variety of host and viral factors.

Hepatitis in humans induced by HBV infection can be acute or chronic, and cirrhosis or hepatocellular carcinoma may be sequelae in persistent-infected patients. Other animal hepadnaviruses have been used as experimental models to study HBV infection at the molecular pathogenesis levels. These animal models include woodchuck hepatitis B virus, Peking duck hepatitis B virus, and ground squirrel hepatitis B virus.

Hepatitis C virus (HCV) was previously considered as the sole etiological viral agent of non-A, non-B viral hepatitis before HEV was discovered (51). HCV, a member of the family *Flaviviridae,* is a linear, positive-sense, single-stranded RNA virus. HCV particles are enveloped. Individuals infected with HCV usually develop persistent viremia, thus HCV infection is commonly transmitted through exposure to blood by parenteral means of drug users. The clinical course of HCV infection is variable, similar to that of HBV infection and HCV-infected human patients may exhibit acute or chronic hepatitis. Hepatocellular carcinoma and/or cirrhosis are reported as a terminal stage of persistent infection in patients with chronic hepatitis C.

Hepatitis Delta virus (HDV) is a defective virus (24). HDV virions are enveloped by the surface antigens of HBV. HDV is a circular, negative-sense, single-stranded RNA virus and is classified as a deltavirus without an assigned family. It requires a concurrent infection of HBV to induce an acute severe hepatitis in humans, and thus shares the parenteral mode of transmission with HBV. HDV-associated hepatitis can be found in individuals with a co-infection with HBV or superinfection of a persistent HBV infection. A high mortality rate or chronic progressive hepatitis in HDV-infected patients is usually associated with coinfections or superinfections of HBV.

Hepatitis G virus (HGV) was discovered in 1995 in individuals with fulminant hepatitis (77). However, HGV is alternatively known as GB virus type C (GBV-C) and currently, GBV-C is preferably used to designate the virus because it has been demonstrated to persistently infect humans without acute or chronic clinical hepatitis. GB was named after the initials of a patient whose serum was the source of the discovery of the virus. GBV-C is a single-stranded RNA virus and is closely related to HCV. GBV-C replicates in lymphocytes, but not in hepatocytes, suggesting it may not be a hepatitis agent. Parenteral and mother-to-infant transmission has been documented as means of GBV-C infection. Interestingly, concurrent infection of GBV-C in humans with human immunodeficiency virus (HIV) infection has been found to prolong the survival rate of the patients with acquired immunodeficiency syndrome (AIDS). It has been demonstrated that *in vitro* coinfection of GBV-C and HIV results in diminished HIV replication, thus may provide novel approaches for the treatment of HIV infection and AIDS.

Hepatitis E Virus

HEV is currently classified as a *Hepevirus*, a member of *Hepeviridae* family (18). HEV is a positive-sense, single-stranded RNA virus. HEV particles are non-enveloped, spherical and 27-34 nm in diameter (9, 69). The genome of HEV is approximately 7.5 kb in length. The genome contains three major open reading frames (ORF); ORF1, ORF2, and ORF3. Open reading frame 1 encodes for non-structural proteins exhibiting activities of methyltransferase, protease, RNA helicase, and RNA-dependent RNA polymerase (20). Open reading frame 2 encodes for structural proteins including a capsid protein. Open

reading frame 3 encodes for a cytoskeleton-associated phosphoprotein which is believed to play a role in post-replication viral assembly (89, 103).

Based on analysis of the complete genomic sequence there are 4 major genotypes of HEV strains (20). Genotype 1 contains the Burmese strain of human HEV as the prototype strain and comprises human HEV strains from Myanmar, India, Japan and South-east Asia. Genotype 1 includes the Sar55 strain from which the capsid protein encoded from the ORF2 region is commonly used as coating antigen for an enzyme immunosorbent linkage assay (ELISA) (2, 69). Genotype 2 includes the Mexican strain as the prototype and comprises several strains found in Nigeria. Genotype 3 includes the United States HEV and comprises the human US-1 and US-2 strains and the prototype swine HEV discovered in the U.S. Genotype 4 comprises the newly-identified human and swine HEV strains from Asia and Europe (72). The recently-discovered prototype U.S. avian HEV is genetically distant from other major genotypes and may represent a new genotype (20, 35). The ORF2 protein of avian HEV is genetically quite distant from the HEV strains in the current 4 major genotypes but reacts to anti-HEV antibodies against the genotype 1 Sar55 strain of human HEV and to genotype 3 swine HEV and US-2 strain of human HEV (28). A recent study of the diversity of HEV genomes demonstrated evidence for quasispecies of HEV during human epidemics (25). Advance in knowledge at the molecular level of HEV has been hampered by the lack of efficient and suitable cell culture systems (19). According to a study of in vitro replication of HEV using primate cell lines, HEV has no cytopathic effect, and replication does not interfere with host cell division (19).

In contrast to the genetic heterogeneity of HEV, it appears that HEV strains belong to a single serotype (8). Anti-HEV antibodies experimentally induced against one HEV strain

are protective against infection by heterologous HEV strains (69). The ORF2 capsid protein from the Sar55 strain of human HEV also reacts to naturally-occurring anti-HEV antibodies in humans and animals that represent different genotypes (8).

HEV is a temperature-sensitive, labile virus under laboratory conditions (76). Freezing-thawing has been reported to greatly affect the infectivity of HEV (8). However, it appears that HEV is relatively resistant to acid and mild alkaline conditions in the intestinal tract since a large amount of HEV is shed in feces and the virus remains infectious when the feces are used as the source of virus inocula in different animal species models (1, 9, 27, 52, 60, 69). HEV was detected in raw sewage collected in the U.S. and European countries including Spain and France (15, 67). The HEV detected in raw sewage from Spain remained infectious to rhesus monkeys (67). The findings imply that HEV may be stable and viable for a certain period of time in variable environments (72).

Hepatitis E Virus in Humans

Human hepatitis E infection induces an acute icteric hepatitis (47, 75). The disease is typically self limiting. Complete remission is expected without chronic damage to the liver (65). Hepatitis E is thought to be transmitted through the fecal-oral route (20). Alternative modes of HEV transmission have been reported and include person-to-person, food-borne, or blood transfusion-associated transmission (14, 22, 53, 76). The mortality is generally low (0.5 to 3%) in people; however, the mortality in pregnant women with fulminant hepatitis E infection may approach 25% (8, 49). Vertical transmission from mother to fetuses also occurs (45).

Hepatitis E virus infection is common in endemic regions in developing countries and is mainly associated with HEV genotypes 1 and 2 (8, 57). Hepatitis E virus infection is rare in industrialized countries and is usually reported in patients who have a history of traveling to endemic countries (68). However, increasing trends of HEV infection have been reported in non-endemic, industrialized countries. Most of these cases are single infections and the patient has no history of traveling to endemic areas (22, 53, 72, 73, 97). Although HEV infection is infrequent in industrialized countries, HEV may be more widespread than previously thought because HEV has been found in human sewage facilities and a high prevalence of anti-HEV antibodies has been demonstrated in particular populations (15, 17, 20, 56, 67, 96).

Experimental studies in nonhuman primates have been used as models for human HEV infection (55, 86, 87, 88). HEV infection in primates is dose dependent (1, 87). Primates with clinical HEV infection had increased levels of liver enzymes (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]), viral shedding in feces, viremia, anti-HEV seroconversion, and necrotizing and lymphocytic hepatitis (55, 74). Primates infected with a low titer of HEV may present with subclinical hepatitis (1, 87). In subclinical HEV infection, primates shed a large amount of HEV in feces without ALT elevation. Delayed anti-HEV antibody responses are also typical of subclinical HEV infection (87).

The pathophysiology of the high mortality present in pregnant women associated with fulminant HEV infection remains obscure. There was no evidence of *in utero* or perinatal infection of offspring borne to pregnant rhesus monkeys infected with human HEV in

various stages of pregnancy (86). Fulminant hepatitis E infection was not reproducible in the primate model (2, 86).

Hepatitis E Virus in Pigs

The first experimental HEV infection in pigs was reported in 1990 (4). Growing pigs were inoculated simultaneously via oral and intravenous routes with a human HEV isolate that was infectious to rhesus monkeys. The pigs developed severe hepatitis with icterus and increased ALT levels and shed HEV virus-like particles in feces.

The prototype swine HEV was discovered in 1997 in the U.S. (61). In contrast to the experimental infection of pigs with human HEV in 1990, natural swine HEV infection did not induce clinical hepatitis in pigs (4, 61). This finding was further confirmed by experimental infection of pigs with the prototype swine HEV and the US-2 strain of human HEV (27). Subsequently, a number of HEV isolates in pigs have been identified and characterized throughout the world (6, 7, 12, 23, 36, 81, 91, 93, 101).

Swine HEV is heterogeneic (40); however, swine and human HEV isolates from the same geographical regions form genetic clusters (6, 7, 29, 36, 65, 73, 91). Serologic evidence indicates that swine HEV is widespread in pigs (61, 93, 101). Furthermore, the prevalence of anti-HEV antibodies is higher in people who are exposed to pigs on a regular basis (17, 58, 96).

Direct evidence of the zoonotic potential of HEV emerged on two recent and separate occasions. Human HEV infection was found in patients who consumed raw liver from a wild boar (54) or raw meat from wild caught deer (93). Comparative analysis of the genetic sequences of HEV identified in human hepatitis E patients and in pig livers sold as food in

grocery stores provided additional evidence that swine HEV in the liver and human HEV in the hepatitis E patients were highly homologous (100). Swine HEV has the ability to cross species barriers to infect nonhuman primates based on experimental inoculation (60). A human HEV strain has also been shown to infect pigs (27). Therefore, pigs are considered an important potential reservoir of HEV.

Full-length infectious clones of swine HEV have been demonstrated to be effective in inducing experimental HEV infection in pigs (37). These infectious clones could be useful for studying the molecular mechanism of cross-species transmission and for determining zoonotic potential of HEV in a swine model.

Contamination of the environment with HEV from pig manure is possible (76). Organ transplantation using pig organs and/or tissues for human recipients may pose an additional risk of xenozoonotic infection of HEV to humans (102).

Hepatitis E Virus in Chickens

Hepatitis E virus was identified in chickens in 2001 and designated as avian HEV (30). The virus was found in flocks of chickens with hepatitis-splenomegaly syndrome in Canada and the U.S. (30, 70). The syndrome affects broiler chickens and laying hens and is characterized by high mortality rates and a drop in egg production. Postmortem examination of affected chickens generally reveals hepato-splenomegaly, regressive ovaries, and serosanguineous abdominal effusion. Histopathological findings include hepatic necrosis and hemorrhage (70). The causative agent (s) responsible for hepatitis-splenomegaly syndrome is currently unknown (70) and there is evidence that avian HEV may be the cause

of the syndrome (30, 79). It was subsequently shown that antibodies to avian HEV were also found in healthy chicken flocks (78, 79).

Avian HEV is comparable morphologically to swine HEV and shares similar genomic arrangement composed of 3 ORFs (30, 38). Avian HEV shares antigenic epitopes in the ORF2 protein with swine and human HEV. However, avian HEV is genetically distant from both human and swine HEV. The nucleotide sequence homology between avian HEV and swine HEV is approximately 60%, suggesting that avian HEV may represent a new genotype of HEV (38). Avian HEV is heterogeneic (39). Serological surveys indicate that avian HEV is enzoonotic in U.S. chicken farms (39, 70). Experimental infection of chickens with avian HEV demonstrated that avian HEV infection is dose dependent (78). Inoculation of chickens with a low titer of avian HEV induced subclinical infection and viremia was not detected. However, viremia and virus shedding in feces appeared as early as 1 week postinfection in chickens inoculated with a high dose of avian HEV. Antibodies to HEV appeared by 4 weeks postinfection and persisted through the termination of the experiment at 12 weeks postinfection (78). Avian HEV found in chickens is transmissible to turkeys but not to rhesus monkeys (38, 78). Chickens and pigs are commonly raised in close proximity. It remains to be determined if there is a link between the avian and swine HEV in the epidemiology of HEV.

Hepatitis E Virus in Other Animals

Naturally-occurring anti-HEV antibodies have been found in a number of domestic animal species including pigs, chickens, rodents, cats and cattle (21, 33, 41, 76, 84, 90). This suggests that there may be several natural reservoirs for HEV. Rats are susceptible to

experimental HEV infection (52). Anti-HEV antibodies are common in rodent populations from different parts of the world (21, 31, 33, 42). This suggests that rodents are a potentially important reservoir for HEV transmission (31). There is indirect evidence that human HEV infection may have originated from the HEV found in domestic rats (31). Rats are also widespread on pig and poultry farms. It is important to better understand if there is a relationship or interaction between pigs and rats and chickens in regards to HEV transmission and epidemiology.

There is one report indicating that HEV may also be found in wild deer (83). Solid experimental evidence does not yet exist to confirm that HEV infects and replicates in wild deer. Livers collected from 2 wild deer in the Midwestern U.S. were tested by RT-PCR and found to be negative for HEV RNA (C. Kasorndorkbua, unpublished data). The RT-PCR used was a universal RT-PCR designed for variant swine HEV isolates (40). Thus, HEV RNA in the deer may not have been amplified since the HEV in the deer may have been a different genotype, if it exists at all. The role of deer in the epidemiology of HEV awaits further investigation.

Pathogenesis

Hepatitis E virus replicates in hepatocytes (11, 13, 26, 27, 95). The cytoplasm of hepatocytes is likely the site of replication (13, 44). Extrahepatic sites of HEV replication also exist and include the small intestines, colon, lymph nodes, tonsil, and spleen (95). The liver is considered to be the main source of HEV particles in feces (69). Pigs infected with either the U.S. human or swine strains of HEV shed the virus in feces for several weeks whereas HEV viremia is transient (27). The HEV genomic RNA has been shown to localize

in the cytoplasm of intrahepatic biliary epithelial cells of rhesus monkeys infected with a virulent human strain of HEV from Myanmar (44). The detection of HEV RNA by *in situ* hybridization (ISH) demonstrated that HEV, either a human or swine strain, may replicate in the cytoplasm of HEV-infected hepatocytes (13, 44). The virions of HEV found in the bile may represent those that replicated in hepatocytes and biliary epithelial cells. The newly-assembled HEV virions may eventually reach the intestinal lumen through the common bile duct. It is logical to assume that the epithelial cells along the intestinal mucosa may be additional sites for HEV replication outside the liver since the intestines (small intestines and colon) have been demonstrated to contain the negative-sense replicative form of HEV genome (95). The replication of HEV in the intestinal mucosa may increase the number of infectious HEV particles found in pig feces which are believed to be the major source of transmission among pigs (26, 98). The portal of entry of HEV to the liver remains unknown. Entero-hepatic circulation may be a candidate route since HEV antigen was found in the mononuclear cells in lamina propia of the intestinal mucosa by ISH (26). The morphology of these HEV RNA-positive mononuclear cells was similar to that of lymphocytes and macrophages (13).

The incubation period of HEV in pigs is relatively short (27). Hepatitis E virus RNA can be detected in feces as early as 1 week postinfection and shedding in feces persists for several weeks. Pigs infected with both human and swine strains of HEV develop anti-HEV antibodies following the viremic stage by 2 weeks postinfection and the antibodies persist for several months (27, 59, 61). HEV infection in pigs induces hepatitis lesions characterized by mild-to-moderate multifocal lymphoplasmacytic infiltrates and focal random hepatocellular necrosis by 7 days postinfection (27). HEV RNA has been found in liver, tonsils, lymph

nodes, spleen, kidney, small intestines, and large intestines from naturally HEV-infected pigs by ISH (13). The detection of HEV antigen further confirmed the tissue distribution of HEV (26). Among the tissues positive for HEV antigen, liver had the greatest intense and extensive amount of staining by immunohistochemistry (IHC).

It is possible that the inflammatory reaction and the necrosis of hepatocytes in the liver are immune-mediated and not the result of direct damage of hepatocytes associated with the replication of HEV. HEV antigen was not detected in degenerative hepatocytes associated with the hepatitic foci in naturally-HEV-infected pigs by IHC (26). This is in agreement with the findings of ISH that did not detect HEV RNA in the cytoplasm of degenerative hepatocytes in naturally HEV-infected pigs (13). The associated infiltrating lymphocytes were positively stained with markers specific for cytotoxic/suppressor properties (74). HEV has been demonstrated to lack cytopathic effect *in vitro* (19). The replication of HEV in primate cell lines showed no destructive effects during cell division (19).

HEV Transmission

Experimental infection of animals with HEV has been consistently achieved by the intravenous route (1, 27, 44, 52, 59, 60, 74, 78, 86, 87, 88). Oral inoculation is less reproducible (55, 69). It has been suggested that swine HEV is naturally transmitted via the fecal-oral route (98). However, the fecal-oral transmission of HEV has yet to be confirmed under experimental conditions. Serological surveys suggest that HEV spreads rapidly among pigs as evidenced by the high prevalence of anti-HEV antibodies in the pig population (12, 93). An experimental study showed that swine HEV was readily transmitted via direct

contact (59). Avian HEV was also transmitted to contact chickens under experimental conditions (78). More work towards understanding the mode or modes of transmission of HEV is needed.

Diagnosis

No clinical disease has been associated with natural infection of pigs with HEV in the field (40, 61). Experimentally-infected pigs had no evidence of clinical disease (27). Current diagnostic methods to confirm HEV infection thus depend on laboratory tests and comprise the detection of anti-HEV antibodies in serum samples, HEV RNA in biological samples (feces, serum, liver, and bile), and HEV particles by electron microscopy (EM). Propagation in cell culture has not yet been successful for use as a diagnostic test or research tool (19, 69).

Currently, the serologic test of choice is an ELISA (69, 82). Major HEV genotype members belong to a single serotype and the antibodies induced by the different HEV strains react well with recombinant capsid antigens produced from 2 strains of human HEV (2, 82). The coating antigen for the ELISA assay used in swine HEV diagnostics is produced from a recombinant capsid antigen of the Sar55 strain of human HEV (27, 61). Specific recombinant ORF2 antigen from avian HEV is used for the detection of antibodies specific for avian HEV (39).

Serological diagnosis can be made by the detection of immunoglobulin (Ig) M or IgG. The presence of anti-HEV IgM indicates early HEV infection which is important in the diagnosis of human infection (2). Anti-HEV IgG has been used primarily on an experimental

basis as a marker for experimental HEV infection of swine HEV in pigs and avian HEV in chickens.

HEV RNA can be detected by a nested reverse transcription polymerase chain reaction (RT-PCR) assay. Nested RT-PCR is time consuming and costly but is a sensitive assay to detect HEV RNA during periods of virus shedding in feces and viremia (27, 59, 60, 64). HEV viremia is transient (1 to 2 weeks). Pigs shed HEV in feces for several weeks thus feces is an appropriate sample for the detection of recent HEV infection in pigs. The primers used in the RT-PCR have been designed to specifically amplify HEV strains in pigs or chickens. Since HEV, particularly in pigs, is heterogeneic, a universal RT-PCR has been developed to amplify all known U.S. swine HEV isolates (40).

The detection of HEV particles by EM is mainly used for diagnosis of human HEV infection. The method is less sensitive than the ELISA and RT-PCR assay because it is difficult to detect the virus particles due to the labile nature of HEV in storage (8, 69).

In primates and humans infected with HEV, elevation of liver-associated enzymes including ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP), and γ-glutamyl transpeptidase (GGT) can be detected in the serum (1, 9, 41, 46, 55, 83, 87). However, experimental infection of pigs with swine HEV was not associated with increase in AST, GGT, sorbital dehydrogenase, and total bilirubin during the 35 day duration of the study (27).

Control and Prevention

Hepatitis E virus is globally ubiquitous in pig populations (8, 93, 98, 99). Subclinical infection of HEV in pigs likely accounts for this high prevalence (99). HEV infection tends

to spread among a population of pigs as soon as maternal anti-HEV antibodies disappear at 8 to 9 weeks of age (61). HEV may originate from the older pigs in the same farms since the virus is shed in large amounts and for a relatively long period in feces (27, 99). These conditions facilitate enzootic infections and constitute the difficulties to control HEV infection on pig farms. Constant and strict biosecurity measures may help prevent HEV spread. In humans, it is clear that strict and proper public hygiene can prevent HEV epidemics in endemic regions. Because HEV is transmitted through the fecal-oral route, the primary preventive measures emphasize effective water treatment and proper sewage disposal (76).

Vaccination may be an option to explore to prevent HEV infections. However, a major obstacle for the production of killed or attenuated HEV vaccine is the lack of an efficient cell culture system for HEV propagation (20, 92). Researchers have investigated the use of recombinant subunit or DNA vaccines (50, 92, 104). Commercially available vaccines have yet to be developed for humans, pigs, or any other animal species (20, 92).

An experiment in nonhuman primates demonstrated that anti-HEV antibodies induced by a recombinant capsid protein of a virulent HEV strain are protective against a heterologous HEV strain (85). In addition, a DNA vaccine expressing the full-length ORF2 which encodes the HEV structural protein induced anti-HEV antibodies in laboratory mice (32). However, challenge infections of susceptible hosts such as nonhuman primates have not been performed to confirm vaccine efficacy (32). Recently, cynomolgus monkeys vaccinated with a plasmid containing a full-length ORF2 sequence of the Burmese strain of human HEV developed anti-HEV antibodies and were protected following challenge infection with the Mexican strain of HEV, a virulent human HEV of different genotype (43).

In these experiments, intradermal or intramuscular routes were used. Oral vaccination using a recombinant capsid protein of HEV expressed in the baculovirus expression system was shown to induce effective protection against HEV infection in cynomolgus monkeys (50). Experiments on vaccine development for protection against HEV infection show promising results; however, the information regarding the duration of acquired protective anti-HEV antibodies is limited (104). The effectiveness of these vaccines awaits trials in humans. Passive immunization with anti-HEV IgG antibodies has been proposed in individuals at risk of HEV infection such as pregnant women or tourists traveling to endemic regions (10, 69). However, passive immunization failed to protect against experimental HEV infection in nonhuman primates (10). No control or eradication measures have currently been attempted to date in pigs, chickens, or other animals.

CHAPTER 3.

USE OF A SWINE BIOASSAY AND A RT-PCR ASSAY TO ASSESS THE RISK OF TRANSMISSION OF SWINE HEPATITIS E VIRUS IN PIGS

A paper published in

Journal of Virological Methods 101:71-78, 2002.

Chaiyan Kasorndorkbua, Patrick G. Halbur, Pete J. Thomas,
Denis K. Guenette, Thomas E. Toth, and Xiang-Jin Meng

Abstract

The objective of this study was to assess the risk of transmission of swine hepatitis E virus (swine HEV) to naïve pigs by inoculation with tissues or feces collected from pigs experimentally infected with swine HEV. Seventy-five, 3-week-old pigs were randomly assigned to 24 groups of 3-4 pigs and inoculated with homogenates of tissues (liver, heart, pancreas, or skeletal muscle) or a suspension of feces from swine HEV-infected pigs collected at 3, 7, 14, 20, 27, or 55 days post inoculation (DPI). Each inoculum was prepared as a 10% suspension (W/V) in PBS buffer and tested by a semi-quantitative RT-PCR for swine HEV RNA and by the swine bioassay. The inoculation route was intravenous for liver, heart, and pancreas and via stomach tube for skeletal muscle and fecal suspension. The liver homogenate inocula and feces collected at 3-7 DPI and 14-20 DPI were positive for swine HEV RNA by RT-PCR. The pigs inoculated with liver homogenates collected at

3-7 DPI and 14-20 DPI developed anti-HEV antibodies and swine HEV RNA was detected in their sera. Pigs inoculated with heart, pancreas, skeletal muscle homogenates or fecal suspensions failed to develop anti-HEV antibodies. These findings suggest that there is a potential risk of transmission of swine HEV via liver tissue from infected pigs in the early stages (3-20 DPI) of infection and the in vitro RT-PCR assay correlates well with the swine bioassay.

Keywords: Swine hepatitis E virus; HEV; swine bioassay; RT-PCR; Pig

1. Introduction

Hepatitis E virus (HEV) is a non-enveloped, single-stranded, positive sense RNA virus and is the causative agent of acute hepatitis E (Purcell, 1996). HEV was classified originally in the family *Caliciviridae* (Purcell, 1996; Reyes, 1997), but was subsequently declassified and designated in an unassigned genus "hepatitis E-like virus" (Pringle, 1998). The disease is considered to be endemic in many developing countries in Asia, Africa and Mexico where the disease usually occurs as epidemics. The natural transmission of HEV is generally believed to be fecal-oral by contamination of drinking water following heavy rains or flooding (Krawczynski, 1993). Sporadic cases of HEV infection have also been reported in industrialized countries such as the United States and European countries where the disease is generally considered as non-endemic and the occurrences are usually associated with travel to endemic countries (Dawson et al., 1992; Herrera et al., 1993; de Groen et al., 1997). Recent cases of hepatitis E infection without a history of travelling to endemic regions have been confirmed in industrialized countries (Schlauder et al., 1998; Schlauder et

al., 1999; Worm et al., 2000). In addition, an isolate of HEV from a pig, designated as a swine HEV, has been identified and shown to be closely related to the two human isolates of HEV (US-1 and US-2) identified in the United States (Meng et al., 1997). Analysis of open reading frames 1 and 2 of the US-1 and US-2 human isolates and swine HEV revealed a relatively high amino acid identity, up to 97% (Meng et al., 1998b). Similar findings have also been reported in pigs from Taiwan with a different strain of swine HEV (Hsieh et al., 1999). Experimental cross species infection has been demonstrated. Swine HEV has been shown to infect rhesus monkeys and a chimpanzee, and the US-2 strain of human HEV has been shown to infect pigs (Meng et. al., 1998a; Halbur et al., 2001). These findings suggest that swine may be an animal reservoir and thus raises a concern of HEV as a potential zoonotic agent (Meng, 2000b; Piper-Jenks et al., 2000).

Seroepidemiological studies of HEV in both endemic and non-endemic regions have shown that anti-HEV antibodies are present in domestic animal species including pigs and captured wild rats (Kabrane-Lazizi et al., 1999; Meng et al., 1999; Favorov, et al., 2000). Pigs as the donor animal species for xenotransplantation have received considerable attention since they are easy to breed, economical to produce, and similar in their physiology and size of organs to humans. Pig liver, pancreatic islets, and heart/valves have been used as organ/tissue for human xenotransplant recipients (Leventhal et al., 1993; Butler, 1998; Yoo and Giulivi, 2000). Xenotransplant procedures which allow pathogenic organism to bypass human defense mechanism due to the immunocompromised condition in human xenograft recipients, would affect the virulence of pathogenic or non-pathogenic agents (Yoo and Giulivi, 2000). A recent study of the experimental infection of swine HEV and the US-2 strain of human HEV in pigs has shown that both strains can infect and replicate in pigs and

the virus was detected in the liver and bile and was also shed in feces (Halbur et al., 2001). Therefore, it is important to assess the risk of transmission of HEV to naïve pigs by inoculation with tissues and feces prepared from known HEV-infected pigs and to evaluate whether or not an in vitro semi-quantitative RT-PCR assay correlates with an in vivo swine bioassay in detecting swine HEV infection. The study was designed to determine if there is a risk of HEV transmission by skeletal muscle tissue via oral inoculation (in an effort to mimic consumption of undercooked pork meat) or by intravenous inoculation of liver or heart or pancreas (to mimic organ/cell transplantation) from HEV-infected pigs to xenograft recipients using a swine model.

2. Materials and Methods

2.1. *Animals*

Seventy-five, 2-week-old, specific-pathogen-free (SPF) pigs (*Sus scrofa domesticus*) were purchased from a commercial farm. All pigs were tested by ELISA assay for swine HEV IgG antibodies. Only seronegative pigs were used for inoculation. The pigs were allowed to acclimate to the research facilities for 1 week prior to inoculation.

2.2 *Inocula*

The inocula were prepared from tissues (liver, heart, pancreas, or skeletal muscle) and feces of SPF pigs experimentally infected with swine HEV from a previous study (Halbur et. al., 2001). Tissues or feces collected at 3 and 7 (3-7), 14 and 20 (14-20), and 27 and 55 (27-55) DPI from HEV-infected pigs were pooled respectively, and used to make the inocula. All tissues and feces were stored at –70°C from the time of necropsy until they were prepared

as inocula. Each inoculum was prepared as a 10% tissue homogenate (w/v) or a 10% suspension of feces (w/v) in PBS buffer (Table 1). All inocula were stored at –70°C until used for inoculation. Each inoculum was tested by a semi-quantitative RT-PCR for swine HEV RNA (see below). The positive control inoculum was a standard infectious pool of swine feces with a $10^{4.5}$ 50 % pig infectious dose (PID_{50}) of swine HEV per ml (Meng et al., 1998b).

2.3 *Detection and semi-quantitative titration of swine HEV RNA in inocula and sera by RT-PCR*

RT-PCR was carried out essentially as described previously (Meng et al., 1998a, 1998b). Briefly, total RNA was extracted from 100 µl of each sample (10% fecal suspension or tissue homogenates) by TriZol reagent (GIBCO/BRL, Gaithersburg, MD). All samples were tested by a nested PCR with primers located in the putative capsid gene (ORF2) (Meng et al., 1998a, 1998b). The first round PCR produced an expected fragment of 404 base pairs with the forward primer F1 (5′-AGCTCCTGTACCTGATGTTGACTC-3′) and the reverse primer R1 (5′-CTACAGAGCGCCAGCCTTGATTGC-3′). For the second round PCR, the forward primer F2 (5′-GCTCACGTCATCTGTCGCTGCTGG-3′) and the reverse primer R2 (5′-GGGCTGAACCAAAATCCTGACATC-3′) produced an expected fragment of 266 base pairs. Total RNA was reverse transcribed with R1 reverse primer and SuperScript II reverse transcriptase (GIBCO/BRL) at 42 °C for 1 hour. The resulting cDNA was amplified by PCR with ampli*Taq* Gold DNA polymerase. The PCR reaction was carried out for 39 cycles of denaturation at 94 °C for 1 minute, annealing at 52 °C for 1 minute, extension at 72 °C for 1.5 minutes, and followed by a final incubation at 72 °C for 7 minutes. The second round PCR

reactions were performed with similar parameters as above with 10 μl of the first round PCR mixture as the template. The amplified PCR products were examined by gel electrophoresis. The virus titer of inocula was calculated and expressed as genome equivalent (GE) per ml of sample (Meng et al., 1998a, 1998b). Our previous studies showed that viremia in swine HEV infected pigs generally occurred from 7 to 20 DPI (Halbur et al., 2001; Williams et al., 2001). Therefore, serum samples of inoculated pigs collected from 7 to 21 DPI were also tested by RT-PCR for swine HEV RNA.

2.4 *Experimental inoculation of SPF pigs and evaluation of clinical signs and lesions*

Seventy-five pigs were randomly assigned into 24 groups of 3-4 pigs and inoculated with different inocula (Table 1). Each group of pigs was housed in a separate room. All pigs were allowed access to food ad libitum. The inoculation route was intravenous for homogenates of liver, heart, and pancreas to mimic xenotransplant procedures. Homogenates of skeletal muscle and fecal suspensions were inoculated orally via stomach tube to determine if there is a risk of swine HEV transmission by consumption of undercooked pork meat or by drinking feces-contaminated water. Homogenates of tissues and suspensions of feces collected from normal SPF pigs served as negative controls and a group of 3 pigs served as uninoculated controls. For positive controls, 3 groups of pigs were inoculated with a $10^{4.5}$ PID_{50}, approximately equivalent to 10^6 GE, of swine HEV via oral drop, stomach tube, or intravenous route, respectively.

Clinical signs were monitored daily until the end of the experiment at 56 DPI. Serum samples were collected weekly for testing for the presence of anti-HEV antibodies by ELISA assay. Pigs were necropsied at the end of the experiment at 56 DPI. Tissues collected from

major organs including liver, gall bladder, small intestines, colon, lymph nodes, spleen, tonsil, heart/heart valves, kidney, and lung were processed by routine procedure for examination for histopathologic changes by hematoxylin & eosin staining.

2.5. Detection of anti-HEV serum antibodies

A standardized ELISA was used to detect anti-HEV IgG antibodies in swine sera. Serum samples were tested prior to inoculation and weekly throughout the study. The ELISA for anti-HEV antibodies in swine was performed essentially as described (Meng et al., 1997, 1998a, b). A purified 55-kDa truncated recombinant putative capsid protein of human HEV strain Sar-55 was used as the antigen. This antigen cross-reacts well with the swine HEV. Peroxidase-labeled goat anti-swine IgG (Kirkgaard & Perry Laboratories, Gaithersburg, MD) was used as the secondary antibody. All serum samples were tested in duplicate. Preimmune and hyperimmune anti-HEV-positive swine sera were included as negative and positive controls, respectively.

3. Results

3.1. Detection of swine HEV RNA in inocula by a semi-quantitative RT-PCR

Swine HEV RNA was detected in the inocula of liver homogenates and feces collected at 3-7 DPI and 14-20 DPI (Table 1). The virus titer of the inocula of liver homogenates collected at 3-7 DPI and 14-20 DPI was approximately 10^4 GE/ml and 10^2 GE/ml, respectively. The virus titer for the 3-7 DPI and 14-20 DPI feces inocula was approximately 10^3 GE/ml. None of the other inocula of tissue homogenates prepared from

heart, pancreas, or skeletal muscle contained detectable swine HEV RNA. The virus titer of the inocula of swine HEV infectious pool was approximately 10^6 GE/ml.

3.2. Clinical evaluation and gross and microscopic lesions

There were no clinical signs in any pigs of any group. Gross and microscopic lesions were unremarkable in pigs necropsied at the end of the experiment (56 DPI).

3.3. Swine bioassay results

Seroconversion to anti-HEV antibodies was used to monitor swine HEV infection in inoculated pigs in this swine bioassay. The positive control pigs inoculated intravenously and the pigs inoculated with liver homogenates collected at 3-7 and 14-20 days post inoculation developed anti-HEV serum antibodies between 3 to 8 weeks post inoculation (Table 1). In the positive control group, only pigs inoculated intravenously seroconverted: one pig became seropositive at 21 days post inoculation and the other two at 28 days post inoculation. Pigs in the other two positive control groups (inoculated orally or via stomach tube) remained seronegative at the end of the study. Pigs in the liver homogenate groups seroconverted between 21 and 35 DPI. Pigs in the 27-55 DPI liver inocula group remained seronegative in the swine bioassay. The 27-55 DPI liver inocula were negative by RT-PCR assay. The 3-7 and 14-20 DPI feces inocula tested positive by RT-PCR assay, but results from the swine bioassay were negative when inoculation was done via stomach tube route. All the other groups remained seronegative through the end of the study at 8 weeks post inoculation (Table 1).

We have previously shown that pigs typically are viremic from 7 to 20 DPI and that seroconversion to HEV antibody is a reliable indicator of swine HEV infection (Halbur et al., 2001). Serum samples collected between 7 to 21 DPI from selected groups (all nine pigs inoculated with swine HEV infectious pool, all nine pigs inoculated with liver homogenate, and all nine pigs inoculated with fecal suspension) were tested by RT-PCR. All but one of the pigs that seroconverted to HEV were also positive for swine HEV RNA in sera by RT-PCR (Table 1). The seropositive pig that was negative for HEV RNA (during 7 to 21 DPI) was from the group intravenously inoculated with the swine HEV infectious pool. There was one pig inoculated with the 14-20 DPI liver homogenate that was positive for HEV RNA at 14 DPI and remained seronegative through 56 DPI.

4. Discussion

Xenotransplantation with swine organs and tissues can potentially offer an alternate solution to the shortage of transplantable human organs. However, xenozoonosis, the inadvertant transmission of swine pathogens from pigs to human xenograft recipients, is of major concern. Previous studies have shown that swine HEV appears to be ubiquitous in pigs, and can infect across species barriers (Meng et al., 1997, 1998a, b, 1999; Meng, 2000a, b; Halbur et al., 2001). Thus, transmission of swine HEV from pig tissues/organs to human xenograft recipients and the potential subsequent transmission of the virus to others such as family members and healthcare workers is possible (Meng, 2000a, b). Since hepatitis E virus is most likely transmitted fecal-orally, there is also growing concern about the possibility of contracting the disease by consuming undercooked pork meat or by drinking swine feces-

contaminated water. Therefore, the objectives of this study were to assess the risks of transmitting swine HEV from infected pigs to naïve pigs and to evaluate the usefulness of an in vitro RT-PCR assay in detecting swine HEV as compared to an in vivo swine bioassay.

The routes of inoculation varied in order to mimic the procedures in xenotransplantation (intravenous), meat consumption (oral), and natural transmission (presumably fecal-oral route). Intravenous inoculation (with feces and liver tissue) was successful, but oral inoculation (with feces and muscle tissue) was not successful in transmission of HEV in this study. Feces and liver tissue were collected from the same pigs, and the liver (with a titer of $10^2 - 10^4$ GE/ml) was determined to be infectious when inoculated intravenously, whereas the feces (with a titer of 10^3 GE/ml) were not infectious when inoculated orally. Even our standard infectious pool (swine feces) of HEV with a titer of 10^6 GE/ml was not adequate to induce infection orally. This observation is consistent with earlier studies with hepatitis A and E viruses in non-human primates (Purcell et al., unpublished). The results suggest that HEV transmission via fecal-oral route requires a much higher dose compared to the intravenous route of transmission. It also suggests that the likelihood of transmitting HEV via consumption of pork from an HEV infected pig is minimal.

It remains unknown how HEV is transmitted under natural conditions. Transmission apparently occurs easily since serological surveys indicate that HEV is ubiquitous in the swine population (Meng et al., 1997; Meng et al., 1999). Previously, it was demonstrated that transmission of HEV by contact exposure of experimentally inoculated pigs with a naïve pig (Meng et al., 1998a). It has also been reported that 8-10 kg pigs were susceptible to

experimental infection with a strain of HEV isolated from a human patient's stool via simultaneous intravenous and oral routes (Balayan et al., 1990).

Many factors may influence the infectivity of HEV infection such as the virus titer, the ratio of infectious to defective viral particles, the inoculation routes, and host factors such as immune status and age at exposure (McCaustland et al., 2000). Our results confirm that virus titer and the routes of inoculation are important for HEV transmission. Host mucosal immunity in the alimentary tract could play a role in diminishing the infectivity of swine HEV via oral inoculation (oral drop or stomach tube). It is possible that IgA could attribute to immune exclusion of the virus on mucosal surfaces (Tizard, 2000; Bourne et. al., 1978). Thus, intravenous inoculation would allow the virus in the inocula to avoid the effect of this host defense mechanism. It remains unclear whether the portal of entry of the virus is via the alimentary tract and if the intestines serve as the initial site of replication before the virus reaches the liver. A recent study suggests that HEV replicates extrahepatically, as negative-strand viral RNA was detected in the small intestines, spleen, lymph node, and tonsil (Williams et al., 2001).

Due to the lack of a suitable cell culture for HEV propagation, the infectivity of HEV can not be determined in vitro. In this study, we attempted to see if a RT-PCR assay could be used to screen swine HEV infectivity in vivo. The findings from the study indicated that the results of inocula tested by RT-PCR assay correlates well with the swine bioassay if the inoculation route was performed intravenously. The inocula tested positive by RT-PCR were infectious in pigs in the swine bioassay via intravenous route, whereas the inocula tested negative by RT-PCR failed to induce seroconversion in the swine bioassay. The data

suggested that RT-PCR assay could be used to detect the presence of swine HEV in pig tissues.

Detection of anti-HEV seroconversion appears to be a reliable indicator for both swine and human HEV infection in experimentally inoculated pigs (Halbur et al., 2001). There was one seropositive pig (in the swine HEV infectious pool group) in the current study from which we were unable to detect viremia between 7 and 21 DPI. It has been reported that HEV viremia may be undetectable until 42 days post-inoculation (Meng et al., 1998a) which may be the case with this pig. There also was one viremic pig that remained seronegative in this study. This pig (inoculated with the 14-20 DPI liver homogenate) might have seroconverted had we allowed the pig to live longer since antibodies may require longer than 56 days to reach detectable levels (Meng et al., 1997; Meng et al., 1998a).

All inoculated pigs remained clinically normal. Since the scope of this study was to determine transmissibility of HEV, we measured seroconversion to HEV in all pigs and performed RT-PCR on sera on selected groups for evidence of virus infection. The pigs all necropsied at the end of this 56-day study had no remarkable gross or microscopic lesions in the liver. This is consistent with our earlier studies showing that swine HEV induced hepatitis lesions that were detectable during the early stages of infection (3 to 20 days post inoculation). The liver lesions were subtle or nearly resolved by 55 days post inoculation in pigs experimentally infected with swine HEV in a previous study (Halbur et al., 2001).

In summary, it was demonstrated that swine HEV can be transmitted to 3-week-old naïve pigs via homogenates of liver from infected pigs in the early stages of disease. Therefore, xenotransplantation with swine HEV-infected pig livers would be a potential source for transmitting the virus to human patients. Other tissues including heart and

pancreas seem to be an unlikely source of swine HEV transmission since the RT-PCR assay was unable to detect swine HEV RNA in those tissues and their inocula also failed to induce seroconversion in the swine bioassay. The data also indicated that there is a minimal risk of transmission of swine HEV by skeletal muscle homogenates. The correlation between the in vitro RT-PCR assay and the in vivo swine bioassay results suggests that RT-PCR may be used as a screening tool for detecting the presence of swine HEV in potential xenograft donor pigs or pig tissues.

Acknowledgements

We thank Dr Prem Paul for technical advice and use of laboratory equipment, Dave Cavanaugh for technical assistance with histopathology, Dr Carles Rosell and Ryan Royer for animal care and necropsy assistances, and Crystal Gilbert for assistance with RT-PCR. We are also grateful to Dr Robert H. Purcell and Dr Suzanne U. Emerson of the National Institutes of Health, Bethesda, Maryland for providing us the standard swine HEV infectious inocula and for recombinant HEV antigen used in the ELISA assay. This study is supported by grants (to P.G. Halbur) from the National Pork Producers Check Off and Iowa Livestock Health Advisory Council, and by grants (to X.J. Meng) from the National Institutes of Health (AI01653 and AI46505).

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Table 1. Risk assessment of transmitting swine HEV from infected pigs to naïve pigs: results of RT-PCR assay and swine bioassay.

Inocula	Inoculation Route	Inocula Titer by RT-PCR (GE/ml)	Seroconversion [a]	HEV RNA in serum [b]
None	Uninoculated	N/A [c]	0/3	NT [d]
Swine HEV infectious pool	Oral drop	10^{6} [e]	0/3	0/3
Swine HEV infectious pool	Stomach tube	10^{6}	0/3	0/3
Swine HEV infectious pool	IV	10^{6}	3/3	2/3
Negative control liver [f]	IV	Negative	0/3	NT
3-7 DPI liver	IV	10^{4}	2/3	2/3
14-20 DPI liver	IV	10^{2}	2/3	3/3
27-55 DPI liver	IV	Negative	0/3	0/3
Negative control feces [f]	Stomach tube	Negative	0/3	NT
3-7 DPI feces	Stomach tube	10^{3}	0/3	0/3
14-20 DPI feces	Stomach tube	10^{3}	0/3	0/3
27-55 DPI feces	Stomach tube	Negative	0/3	0/3
Negative control heart [f]	IV	Negative	0/3	NT
3-7 DPI heart	IV	Negative	0/4	NT
14-20 DPI heart	IV	Negative	0/3	NT
27-55 DPI heart	IV	Negative	0/3	NT
Negative control pancreas [f]	IV	Negative	0/3	NT
3-7 DPI pancreas	IV	Negative	0/4	NT
14-20 DPI pancreas	IV	Negative	0/4	NT
27-55 DPI pancreas	IV	Negative	0/3	NT
Negative control muscle [f]	Stomach tube	Negative	0/3	NT
3-7 DPI muscle	Stomach tube	Negative	0/3	NT
14-20 DPI muscle	Stomach tube	Negative	0/3	NT
27-55 DPI muscle	Stomach tube	Negative	0/3	NT

[a] Anti HEV antibodies detected by ELISA, number positive pigs/number tested.
[b] Serum samples tested by RT-PCR at 7, 14, and 21 DPI, number positive pigs/number tested.
[c] Not applicable.
[d] Samples not tested.
[e] Approximately equivalent to $10^{4.5}$ PID_{50}.
[f] Negative control tissues and feces were collected from normal SPF pigs that were negative for swine HEV.

CHAPTER 4.

EXPERIMENTAL INFECTION OF PREGNANT GILTS WITH SWINE HEPATITIS E VIRUS

A paper published in

The Canadian Journal of Veterinary Research 67:303-306, 2003.

Chaiyan Kasorndorkbua, Brad J. Thacker, Patrick G. Halbur, Denis K. Guenette, Ryan M. Buitenwerf, Ryan L. Royer, and Xiang-Jin Meng

Abstract

To determine the effect of swine hepatitis E virus (HEV) infection on pregnant gilts, their fetuses, and offspring, 12 gilts were intravenously inoculated with swine HEV. Six gilts, who were not inoculated, served as controls. All inoculated gilts became actively infected and shed HEV in feces, but vertical transmission was not detected in the fetuses. There was no evidence of clinical disease in the gilts or their offspring. Mild multifocal lymphohistiocytic hepatitis was observed in 4 of 12 inoculated gilts. There was no significant effect of swine HEV on fetal size, fetal viability, or offspring birth weight or weight gain. The offspring acquired anti-HEV colostral antibodies but remained seronegative after the antibodies waned by 71 days of age. Swine HEV infection induced subclinical hepatitis in pregnant gilts, but had no effect on the gilts' reproductive performance, or the

fetuses or offspring. Fulminant hepatitis associated with HEV infection was not reproduced in gilts.

Summary of short communication

Human hepatitis E virus (HEV) is the causative agent of acute non-A, non-B, and icterus-inducing hepatitis in humans and is characterized as a non-enveloped, single-stranded, positive sense RNA virus (1). Hepatitis E virus is considered to be enterically transmitted via the fecal-oral route. Swine HEV, first isolated from a pig in Illinois, is closely related to two human isolates of HEV (US-1 and US-2) identified in the United States (U.S.) (2). Cross species infection has been experimentally demonstrated; swine HEV infected rhesus monkeys and a chimpanzee, and the US-2 strain of human HEV infected pigs (3, 4). These findings infer that swine may be an animal reservoir for HEV, raising concern that HEV is a potential zoonotic or xenozoonotic agent (5). Swine HEV is reportedly ubiquitous in the U.S. swine population (2). Furthermore, occupational exposure to swine such as with swine farmers or veterinarians poses a higher risk of HEV infection among these individuals, suggesting the possibility of animal-to-human transmission (6, 7).

The course of hepatitis E in humans is self-limiting and chronic illness is not observed (1). Overall case mortality is low, ranging from 0.2% to 4%, although high mortality rates of 10% to 25% have been reported in pregnant women suffering from fulminant hepatitis associated with HEV. This particular presentation of hepatitis E mostly occurs in the third trimester of pregnancy (8, 9). Explanation for this phenomenon is still obscure. Vertical transmission of HEV via intrauterine infection was suggested as HEV RNA was detected in cord blood samples from infants born to mothers affected with acute

fulminant hepatitis (10, 11). Accordingly, an animal model would be useful to further the understanding of HEV-induced fulminant hepatitis in pregnant women. Non-human primates (cynomolgus macaques, chimpanzees and rhesus monkeys) are susceptible to HEV infection and have been widely used in experimental models (4, 12). However, pregnant rhesus monkeys inoculated intravenously with human HEV strain SAR-55 from Pakistan did not exhibit the characteristics of the fulminant hepatitis disease as seen in pregnant women. Neither a fatal effect of HEV infection on the mother or the fetuses, nor neonatal infection, was found in the study (3).

Since the discovery of swine HEV, experimental studies of swine HEV infection in growing pigs have been well described (3). Lack of an animal model for reproducing fulminant hepatitis E in pregnant women and the need for information regarding the effect of HEV infection in pregnant swine prompted us to investigate the effect of swine HEV infection in pregnant gilts during late gestation on dams, fetuses and offspring, and to determine if the disease pattern of fulminant hepatitis E in pregnant women can be reproduced in pregnant swine.

Eighteen swine HEV-seronegative gilts (*Sus scrofa domesticus*), originating from the Iowa State University specific-pathogen-free herd were used and randomly assigned to swine HEV-inoculated group (n=12) or sham-inoculated control group (n=6). The experimental procedures were reviewed and approved by the Iowa State University Committee on Animal Care. The swine HEV inocula contained a titer of $10^{4.5}$ 50% pig infectious dose (PID_{50}) per mL, which was equal to the titer used in a previous experimental infection of swine HEV study in growing pigs (3). Twelve gilts were intravenously inoculated via an ear vein at 78 to 80 d of gestation. Clinical observations (appetite, lethargy, icterus, or diarrhea) were

conducted daily throughout the study and gilt rectal temperatures were measured for 14 d after inoculation. Five to 6 gilts (4 inoculated and 1 or 2 controls) were euthanized by intravenous administration of an overdose of sodium pentobarbital on 3 separate days as follows; 91 d of gestation (12 d post inoculation [DPI], 1 control and 4 inoculated) 105 d of gestation (26 DPI; 2 controls and 4 inoculated), or at 17 to 19 d after farrowing (55 DPI; 2 controls and 4 inoculated). One control gilt that had gone into estrus again at 21 d post-service and was reserviced immediately, was necropsied at 46 DPI or 81 d of gestation. Four, 8- to 10-day-old piglets from each of the 6 sows that farrowed were necropsied at 46 DPI. Gross examination of the reproductive tract and internal organs of the gilts and offspring was performed. At necropsy, the number of fetuses, fetal viability and condition, fetal weight, and fetal crown-rump length were recorded. A set of tissues including liver, gall bladder, uterus, ovary, salivary gland, duodenum, jejunum, ileum, colon, pancreas, tonsil, spleen, lymph nodes, heart, skeletal muscle, lung, and kidney were collected from each gilt for microscopic examination, using hematoxylin and eosin staining. A set of tissues collected from each pig included liver, brain, thymus, tonsil, spleen, lymph nodes, lung, kidney, heart, duodenum, jejunum, ileum, and colon. The same types of tissues (excluding lymph nodes) as collected from the pigs, plus the umbilical cord, were collected from fetuses for microscopic evaluation. Offspring were weighted at birth and periodically thereafter to evaluate weight gain. All surviving pigs were weaned at 18 d of age and pigs from control gilts were mixed with those from inoculated gilts. Blood samples from these pigs were periodically collected for HEV serology until 102 d of age.

Blood samples were collected from the gilts prior to and throughout the study for HEV serology, HEV RNA detection by a nested reverse transcriptase-polymerase chain

reaction (RT-PCR) assay, and liver chemistry profile (alkaline phosphatase, aspartate aminotransferase, gamma-glutamyl transferase, sorbitol dehydrogenase, and total bilirubin) by an automated analyzer (Hitachi 912; Roche, Indianapolis, Indiana, USA). Fecal samples were also collected at the same time to be tested for HEV RNA by RT-PCR. Overall, samples for RT-PCR testing included serum, feces, liver tissues, and bile obtained from the gilts, and serum and pooled tissues (liver, lung, kidney, spleen, and small intestines) obtained from 2 fetuses (both normal or one normal and one mummified fetus with crown-rump length more than 15 cm.) per litter. A nested RT-PCR technique was performed as previously described (3). The enzyme linked immunosorbent assay (ELISA) for swine anti-HEV antibodies was performed as previously documented (2). The cut-off absorbance value used for positive samples was 0.3.

A commercial software package was used to perform statistical analyses (Statistix, version 4.0; Analytical software, Tallahassee, Florida, USA). Proportional reproductive data were tested by Fisher's exact test (proportion of normal, small, stillborn or mummified fetuses/pigs). Other data (rectal temperatures, liver chemistry profile values, fetal weight and length, offspring birth weight, and weight gain and litter size) were calculated as mean ± standard deviation (s). Data were tested for statistical difference between inoculated and control groups by using an analysis of variance (ANOVA). For all analyses, values of $P < 0.05$ were considered significant.

There were no clinical signs or fever observed in the inoculated gilts or their piglets (data not shown). Liver chemistry profile values were no different between inoculated and control gilts (data not shown). There were no significant differences between inoculated and control groups with regard to fetal weight and length, or offspring birth weight and weight

gain (data not shown). None of the reproductive parameters from inoculated gilts were significantly different from control gilts (Table I). Although, the percentage of mummified fetuses in inoculated gilts was higher than control gilts, based on crown-rump length, the majority of these fetuses died prior to inoculation. Overall, litter size was relatively high for gilts and at necropsy many of the mummified fetuses were clustered together in one segment of the uterus, suggesting that fetal death occurred due to intra-uterine crowding. There were no significant gross lesions in any of the gilts, fetuses or piglets. Mild multifocal lymphohistiocytic hepatitis associated with occasional individual hepatocyte necrosis was found in 4 of 12 inoculated gilts. Four inoculated gilts (including 2 of the gilts with hepatitis lesions) had mild multifocal lymphohistiocytic interstitial nephritis. There were no remarkable microscopic lesions in any tissues obtained from piglets or fetuses.

All except 1 of the inoculated gilts (7 of 8 gilts) seroconverted to anti-HEV by 19 DPI. Four inoculated gilts were necropsied at 12 DPI, hence their serum samples were not available for ELISA testing thereafter. All 8 remaining gilts subsequently acquired anti-HEV antibodies by 26 DPI and all 4 gilts that were allowed to farrow continued to be seropositive through to 47 DPI. All control gilts remained seronegative throughout the study. Detectable HEV antibodies were found in pigs from HEV-inoculated dams, when first samples at 10 d of age. A proportion of the pigs continued to be seropositive until 61 d of age and all pigs were seronegative thereafter (102 d of age) (Table II). Results of the RT-PCR of samples obtained from swine HEV-inoculated gilts and their fetuses are summarized in Table III. All samples obtained from control gilts and their fetuses contained no detectable level of swine HEV RNA. Since experimental swine HEV infection is readily detected by anti-HEV ELISA assay, fecal samples obtained from piglets were not tested for HEV RNA (3, 14)

Our findings demonstrated that there was no adverse effect of intravenous inoculation of pregnant gilts with U.S. swine HEV. Although the gilts shed swine HEV RNA in feces, there was no evidence of vertical transmission from dams to their fetuses. Pigs born to swine HEV-infected dams acquired anti-HEV Immunoglobulin (Ig) G antibodies passively from colostrum and the passive antibodies persisted until the pigs were approximately 2 mo old. Based on our results, HEV-free pigs can be derived from HEV-infected dams by early weaning and segregation of the pigs from their dams.

Fulminant hepatitis E was not reproduced in pregnant rhesus monkeys intravenously inoculated with $10^{5.5}$ 50% monkey infectious dose (13, 15). Similarly, fulminant hepatitis E was not reproduced in pregnant swine inoculated intravenously with swine HEV. In the current study, we used swine HEV inocula with a titer of $10^{4.5}$ PID_{50}. This inoculating dose has been repeatedly used and determined to be effective for inducing experimental HEV infection and mild hepatitis lesions in pigs (3, 14). It is apparent that inducing experimental HEV infection via intravenous inoculation is more reproducible than via oral inoculation in non-human primates and swine (14, 16). Perhaps, higher doses could result in clinical disease in the pregnant gilt model.

The characteristics of hepatitis lesions observed in the inoculated gilts are consistent with those previously documented in growing pigs and are reportedly milder in pigs inoculated with swine HEV than in pigs inoculated with human HEV (3). With regard to the interstitial nephritis lesions found in 4 of 12 inoculated gilts, the lesions are unlikely related to swine HEV infection since there was no such lesion found in growing pigs inoculated with swine HEV (3). These nephritis lesions may be attributable to previous episodes of ascending

bacterial infection of the lower urinary tract. Recurrent lower urinary tract infections and nephritis in breeding aged pigs is common (17).

Acknowledgments

The authors thank Drs. Robert H. Purcell and Suzanne U. Emerson of the National Institutes of Health, Bethesda, Maryland for kindly providing the SAR-55 antigen and the swine HEV inocula. We also thank Crystal Gilbert for her assistance in PCR testing of swine HEV RNA. This research was funded by grants from the National Pork Board Pork Check Off Dollars, and the Iowa Livestock Health Advisory Council.

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Table I. Reproductive parameters from swine hepatitis E virus (HEV) inoculated gilts versus control gilts.

Parameter	Inoculated gilts	Control gilts
Total fetuses or pigs per litter	11.8	12.5
% Normal	73.9	76.0
% Small or stillborn	7.7	12.0
% Mummified fetuses	NA	NA
<15 cm long (preinoculation)	17.6	9.3
<15 cm long (postinoculation)	0.7	2.7

Table II. Anti-Hepatitis E virus (HEV) immunoglobulin (Ig) G antibodies enzyme linked immunosorbent assay (ELISA) in pigs born from inoculated and control gilts.

	Number seropositive/Number tested (O.D. mean ± *s*)[a]	
Age (days)	**Inoculated gilts**	**Control gilts**
10	13/16 (0.795 ± 0.502)	0/8 (0.000 ± 0.000)
18	17/20 (0.880 ± 0.501)	0/8 (0.069 ± 0.013)
32	14/22 (0.512 ± 0.314)	0/8 (0.151 ± 0.223)
47	8/22 (0.261 ± 0.153)	0/8 (0.043 ± 0.039)
61	2/22 (0.176 ± 0.090)	0/8 (0.071 ± 0.026)
71	0/22 (0.138 ± 0.052)	0/8 (0.066 ± 0.008)
87	0/22 (0.121 ± 0.032)	0/8 (0.080 ± 0.008)
102	0/22 (0.113 ± 0.028)	0/8 (0.139 ± 0.108)

Pigs were weaned at 18 days of age. Pigs from control gilts were mixed with those from inoculated gilts at weaning.
s – Standard deviation
[a] ELISA O.D. cut-off value = 0.3

Table III. Presence of swine hepatitis E virus (HEV) RNA detected by nested RT-polymerase chain reaction (RT-PCR) in samples from swine HEV-inoculated gilts and their fetuses.

Sample	Number positive/number tested (DPI)			
	0	6	12	26
Gilts				
Serum	0/12	2/12	3/11[a]	0/6[a]
Feces	0/12	12/12	12/12	0/8
Liver	NT	NT	4/4	0/4
Bile	NT	NT	2/4	0/4
Fetuses				
Serum	NT	NT	0/8[b]	0/8
Pooled tissues[c]	NT	NT	0/8	0/8

DPI – Days post inoculation
NT – Not tested, samples not tested on this DPI due to no necropsy
[a] Serum samples of 1 or 2 inoculated gilts were not available at this DPI.
[b] Number of litters (2 fetuses each) positive for swine HEV RNA/total litters tested
[c] Liver, lung, kidney, spleen, and small intestines

CHAPTER 5.

ROUTES OF TRANSMISSION OF SWINE HEPATITIS E VIRUS IN PIGS

A paper published in

Journal of Clinical Microbiology 42:5047-5052, 2004.

Chaiyan Kasorndorkbua, Denis K. Guenette, Fang F. Huang,

Pete J. Thomas, Xiang-Jin Meng, Patrick G. Halbur

Abstract

Hepatitis E virus (HEV) is believed to be transmitted by the fecal-oral route in pigs. To date, in experiments, HEV has been transmitted successfully by the intravenous or intrahepatic route. To assess the route of HEV transmission, 27 pigs were separated into nine groups of three pigs. Positive-control pigs were inoculated intravenously with swine HEV and served as the source of HEV for the other groups. Uninoculated contact pigs were placed in the positive-control group. On three consecutive days, naïve pigs were inoculated using samples collected from the positive-control pigs at 9, 10, and 11 days postinoculation. The tonsils and nasal mucosa of each positive-control pig were swabbed and that swab was used to rub the tonsils, nasal and ocular mucosa of naïve pigs. The positive-control pigs were also injected with bacterin, and the same needle was used to immediately inject naïve pigs. Feces were collected from positive controls and fed by oral gavage to naïve pigs. Weekly fecal and serum samples from each pig were tested for anti-HEV antibodies and HEV RNA. All

positive-control pigs shed the virus in feces; two were viremic and seroconverted to anti-HEV. All contact control pigs shed the virus in feces, two seroconverted and one became viremic. One of three pigs in the fecal-oral exposure group shed the virus in feces and seroconverted. Pigs exposed to the contaminated needles or the tonsil/nasal secretions swabs remained negative. This is the first report of experimental fecal-oral transmission of HEV in swine.

Introduction

Hepatitis E virus (HEV) is an important cause of enterically transmitted, non-A, non-B hepatitis in humans. The virus is a non-enveloped, single-stranded, positive sense RNA virus (34). HEV has recently been classified as the prototype member in the *hepevirus* genus of the family *Hepeviridae* (9). The disease caused by HEV is typically characterized as a self-limiting acute hepatitis with low mortality (20). However, severe hepatitis has been reported in pregnant women with up to 25% mortality (20).

HEV in humans is believed to be transmitted primarily by the fecal-oral route (21, 34). HEV is endemic or epidemic in certain regions of the world including parts of Africa, Asia, and Mexico. Epidemics within endemic regions are usually associated with heavy rains or flooding in areas that lack proper drinking water sanitation (19). Individuals from non-endemic regions who acquire HEV infection often have a history of traveling to developing countries where HEV is endemic (7, 14, 33).

Recent sporadic human HEV infections in people who had not traveled to countries where HEV is endemic led to the discovery of novel HEV isolates in industrialized regions, such as the United States, Europe, Taiwan, and Japan. Sequence analyses revealed that these

HEV isolates are genetically divergent (11, 30, 31, 35, 36, 44, 48). The human and swine HEV isolates from industrialized countries are genetically clustered together in the same genotype (either genotype III or IV) raising concerns of hepatitis E as a zoonotic disease (3, 10, 15, 24, 29, 32, 35, 37, 40, 45, 47). Serological surveys of humans who are in close contact with pigs, such as swine veterinarians and pig handlers, showed an increased prevalence of anti-HEV antibodies in these occupational groups suggesting potential pig-to-human transmissions (8, 27, 43).

Pigs have been experimentally infected with a genotype III human HEV and swine HEV, and the HEV-infected pigs shed the viruses in feces for several weeks (13). Direct evidence of zoonotic HEV transmission has recently been reported in Japanese patients who acquired hepatitis after consumption of uncooked pig livers (22) or consumption of raw meat from wild deer (38). On the basis of the sequence available, the swine HEV isolate detected in a raw pig liver sold in a grocery store was genetically identical to a human HEV isolate recovered from one of the Japanese hepatitis E patients (47).

The natural route(s) of swine HEV transmission in pigs remains unknown. Swine HEV can be transmitted experimentally via direct contact with infected pigs (25). Repeated direct daily contact among pigs reared in confinement buildings may enhance the spread of swine HEV. Pigs housed in the same pen are exposed to saliva, nasal secretions, urine, and feces of multiple pen mates repeatedly each day. Experimental transmission of HEV to naïve pigs via feces collected from swine HEV-infected pigs was achieved when inoculated intravenously, but not when the pigs were inoculated orally with an equivalent dose (17). Extrahepatic sites of HEV replication exist and include the intestinal tract (42). It is therefore

logical to assume that, under natural conditions, swine HEV is transmitted via the fecal-oral route as is thought to be the case in human HEV infections (10, 46).

HEV viremia is transient and lasts only 1-2 weeks, whereas fecal virus shedding may persist for up to 7 weeks (13, 25). Repeated use of needles for drug administration or vaccination is commonly practiced in swine health management. Even though HEV viremia is transient (1-2 weeks), it is possible that blood contamination of needles from viremic pigs may also be a means for HEV to spread among pigs on and between farms. The objective of the present study was to determine if swine HEV transmission occurs via exposure to (i) tonsil and nasal secretions from infected pigs (ii) repeated use of contaminated needles, and (iii) oral consumption of feces from infected pigs.

Materials and Methods

Virus inocula and pigs. The swine HEV inocula used in the present study was prepared from feces collected from pigs intravenously infected with the prototype strain of swine HEV (24). The inocula contained a HEV titer of $10^{4.5}$ 50% pig infectious dose per ml, which is equivalent to approximately 10^6 genome equivalent (GE) per ml (26). The inocula were kept at -80° C until used. Twenty-seven, 3-week-old, specific-pathogen-free pigs (*Sus scrofa domesticus*) were used in this study. All pigs were confirmed to be free of swine HEV in feces by nested RT-PCR prior to inoculation (26).

Experimental design. The experimental design consisted of one negative control, three sham-inoculated, and five exposure groups (Table 3). Each group contained three pigs and was housed separately. In the exposure groups (groups 3, 4, and 5), each pig was in a different, individual pen to avoid direct contact with body secretions or excretions from other

pigs in the same groups. Three pigs were intravenously inoculated with the swine HEV inocula and served as positive controls and virus shedders (group 1). Three pigs were non-inoculated and served as negative controls (group 6) and as the source for swine HEV-negative inocula used in the three sham exposure groups. Three uninoculated pigs were housed in the same pen containing the three-positive control pigs to serve as direct-contact controls (group 2). The three exposure routes chosen in the study were designed to evaluate three potential transmission routes likely to occur under current swine production practices: (i) tonsil and nasal secretions, (ii) repeated use of needles, (iii) fecal-oral route.

In the tonsil and nasal secretions exposure group (group 3), sterile plastic Dacron swab applicators (Medical Packaging Corp., Camarillo, Calif.) were used to collect tonsil and nasal secretions from each of the three pigs (a separate swab for each pig) in the positive-control group (virus shedders) by repeatedly and aggressively rubbing the palatine tonsil surface and the nasal mucosal surfaces, respectively. Each of the contaminated swabs was then placed in a separate sterile plastic container and applied to one of the naïve pigs in the exposure group by rubbing the palatine tonsil surface, nasal mucosal surfaces, and palpebral and bulbar conjunctiva, respectively.

In the needle exposure group (group 4), a 1-in.-long, 18-gauge, hypodermic needle and a 3-ml syringe were used to vaccinate three positive control pigs intramuscularly in the neck with 2 ml of *Mycoplasma hyopneumoniae* bacterin (Respisure, Pfizer). The needle was then removed and placed in a sterile plastic bag to repeat the administration of the vaccine to three naïve pigs in the needle exposure group.

In the fecal-oral exposure group (group 5), 15 g of fresh feces collected from each of the shedder pigs was pooled and mixed, and then 15 g of pooled feces was administered by

oral gavage to each of the three naïve pigs in the fecal-oral exposure group. The pigs were monitored for 5 minutes to assure that no regurgitation occurred after the gavage.

The three inoculation procedures were repeated for three consecutive days (9 to 11 days post inoculation [dpi] of the positive-control shedder group). Three sham-inoculated controls (group 7, 8, and 9) were inoculated in similar manners to the corresponding exposure groups, except that the sham inocula were obtained from three swine HEV-free negative-control pigs (group 6). To prevent potential cross contamination via people moving from the shedder group into exposure groups, two separate teams were involved in the collection of exposure inocula and inoculation.

The serum samples and fecal swabs from the positive control shedders were collected daily from 3 to 14 dpi and stored at -80° C for the detection of HEV RNA by qualitative and quantitative RT-PCR. The tonsil and nasal swabs, serum samples, and fecal swabs collected on 9, 10, and 11 dpi were tested for HEV RNA by RT-PCR, and the positive samples were further tested by quantitative real-time RT-PCR. Fecal swab and blood samples were collected weekly from all pigs until the end of the study at 56 dpi. All fecal and serum samples were stored at -80° C until tested.

Detection of swine HEV RNA by nested RT-PCR. RNA extraction was performed by the modified spin column method (QIAamp; QIAgen, Chatsworth, Calif.) as previously described (2). Portions (140 µl) of fecal suspension or serum samples were used for RNA extraction. The RNA extract was then immediately used for the reverse transcription reaction and cDNA synthesis in a reaction containing 11.5 µl of RNA, R1 reverse primer, and Superscript II reverse transcriptase (GIBCO-BRL) at 42° C for 1 hour. A nested RT-PCR assay specific for the prototype United States strain of swine HEV was performed as

previously described (17). The first-round PCR primers were the forward primer F1 (5′-AGCTCCTGTACCTGATGTTGACTC-3′) and the reverse primer R1 (5′-GCTACAGAGCGCCAGCCTTGATTGC-3′) and yielded a PCR product of 404 bp. The second-round PCR primers included the forward primer F2 (5′-GCTCACGTCATCTGTCGCTGCTGG-3′) and the reverse primer R2 (5′-GGGCTGAACCAAAATCCTGACATC-3′) and produced a PCR product size of 266 bp. Ten microliters of cDNA was used as the template for the first round of PCR in a 100-μl PCR mixture. The first-round of PCR was initiated with the activation of ampli*Taq* Gold DNA polymerase at 95° C for 9 min. There were 39 cycles in the first round of PCR, with 1 cycle consisting of denaturation (1 min at 94° C), annealing (1 min at 52 ° C), and extension (1.5 min at 72 ° C). The first round of PCR concluded with a final incubation step (7 min at 72 ° C). Similar PCR parameters were used for the second round of PCR. The amplified PCR products were examined by 1% agarose gel electrophoresis. The PCR products were directly sequenced to confirm that they are indeed swine HEV sequences.

Detection of Immunoglobulin G anti-HEV antibodies by ELISA. Serum samples were tested by an enzyme-linked immunosorbent assay (ELISA) using a purified 55-kDa truncated recombinant capsid protein of human HEV strain Sar-55 as previously described (24, 26). Each serum sample was tested twice. The final optical density values were averages of the two results. Preimmune and hyperimmune swine sera were used as negative and positive controls, respectively.

Quantitative real-time RT-PCR. Representative samples of feces, tonsil and nasal secretions, and serum were collected daily from three shedder pigs in the positive-control

group for three consecutive days during the period of exposure inoculation (9, 10, and 11 dpi). Swabs of pooled fresh feces (used as inocula) were resuspended in 1-ml portions of sterile phosphate buffer saline (PBS). Swabs of tonsil and nasal secretions were resuspended in 1-ml portions of sterile PBS.

All representative samples were subjected to a quantitative real-time RT-PCR assay. A standard curve for the quantification of swine HEV genome was generated using an in vitro swine HEV ORF2 cDNA plasmid as previously described (42). In vitro-transcribed RNA was quantitatively measured by a spectrophotometer. Serial 10-fold dilutions from 100 ng to 10 fg were made and subjected to quantitative PCR. All representative samples of three exposure inocula on 9, 10, and 11 dpi were tested in triplicate using the iCycler system (Bio-Rad). SYBR Green 1 was used to detect PCR product. The amount of viral RNA in the samples was derived from the standard curve using the mean of the cycle threshold values generated. For the amount of viral RNA in a given sample, viral genome copy numbers were calculated according to an equation published elsewhere (5).

Results

The three positive-control pigs inoculated intravenously with the swine HEV stock shed relatively large amounts of viruses in feces during the period (9 to 11 dpi) that the experimental transmission and exposure were performed (Pig A, B, and C in Tables 1 and 2). However, viremia was detected in only two of the pigs (pig B and C) at the times tested, and these pigs seroconverted to immunoglobulin G anti-HEV antibodies by the end of the study (Table 1). Two of the three positive-control pigs exhibited fecal virus shedding during the entire exposure period (on 9 to 11 dpi) (Table 2). Pig A shed virus in feces on 4, 7, 8, and 10

to 14 dpi, pig B shed virus in feces on 4 and 7 to 14 dpi, and pig C shed virus in feces on 3 to 5 and 7 to 14 dpi. Only pig B had three consecutive days of viremia during the exposure period from 9 to 11 dpi (Table 2). Viremia was detected in pig B on 5, 6, and 8 to 14 dpi (Table 1). The HEV genomic titers in serum samples obtained from pig B were 2.8 X 10^6, 4.4 X 10^6, and 6.6 X 10^6 copies per ml of sera on 9, 10, and 11 dpi, respectively (Table 2). Viremia was not detected in pig A or pig C during the exposure period (9 to 11 dpi) or at any time prior to 14 dpi (3 to 13 dpi daily). Pig A was not viremic at any time tested from 14 to 56 dpi; however, viremia was detected in pig C on 14 and 21 dpi (Table 1).

The results of quantitative real-time RT-PCR for HEV genome during the period of exposure are summarized in Table 2. The HEV genomic titers in fecal samples from pig A were 2.7 X 10^5 and 10.5 X 10^6 copies per g of feces on 10 and 11 dpi, respectively (fecal sample from this pig was negative for HEV RNA by RT-PCR on 9 dpi). The genomic titers in fecal samples of pig B were 2.9 X 10^6, 3.7 X 10^6, and 10.5 X 10^6 copies per g of feces on 9, 10, and 11 dpi, respectively. Pig C had 3.8 X 10^6, 6.2 X 10^6, and 13.2 X 10^6 copies per g of feces on 9, 10, and 11 dpi, respectively. The tonsil and nasal secretion swabs collected from the shedder pigs (A, B, and C) were negative for HEV RNA during the period of exposure inoculation (9 to 11 dpi).

Although all three contact control pigs shed the virus in feces, only two of these pigs seroconverted by the end of the study on 56 dpi (Table 3). One of the two seropositive pigs was also viremic on 21 dpi. Only one of three pigs in the fecal-oral exposure group shed the virus in feces (from 21 to 35 dpi) and seroconverted by 56 dpi. Viremia was not detected in that pig on the days tested (14, 21, 28, 35, 42, 49, and 56 dpi). Pigs in the other exposure and

sham inoculation groups remained negative for swine HEV RNA and anti-HEV antibodies throughout the study.

Discussion

This is the first report of successful transmission of HEV in pigs by the fecal-oral route. Of the three possible routes of HEV transmission (tonsil and nasal secretions, contaminated needles, and fecal-oral exposure) investigated under the conditions of this study, the fecal-oral route was the only route of exposure through which HEV transmission was achieved. However, it remains unclear if the fecal-oral route of exposure accounts for the high incidence of HEV infection in the global pig population, since we achieved transmission in only one of three pigs by the fecal-oral route.

In contrast to the contact control pigs, and to the way pigs are typically raised in the field, the three pigs in the fecal-oral exposure group were placed in individual pens in the same room to decrease the likelihood of exposure to feces and urine from other pigs in the room. Aerosol transmission would not have been prevented by the room and pen design. Since transmission did not occur from pig to pig within the room, aerosol transmission is not likely. The flooring of the animal pens used in the study was raised, plastic-coated metal wire decks which accumulate minimal amounts of feces. In contrast, under field conditions, most pigs are raised in confinement facilities that have 25 to 200 pigs per pen and a combination of solid and slatted concrete flooring, which generally allows for more feces to accumulate and thus for repeated exposure to feces from multiple pigs.

Experimental HEV infection by oral inoculation in nonhuman primates has reportedly been less reproducible than intravenous inoculation (1). Furthermore, an attempt to reproduce

swine HEV infection by oral inoculation in pigs was unsuccessful with a single dose of a standard swine HEV stock that was infectious when given intravenously (17). This suggests that swine HEV infection in pigs requires either a higher titer or repeated exposure to initiate infection via the fecal-oral route. Direct and repeated contact among large numbers of pigs within the same pen would increase the likelihood of repeated exposure to pigs excreting a high dose of HEV in feces. It is obvious that the infectious dose of HEV required for successful HEV transmission is different for the intravenous and oral routes of exposure. It has been demonstrated that a HEV titer of 10^2 GE was sufficient to induce HEV infection in pigs when the virus was inoculated intravenously on a single day (17); however a higher titer of more than 10^6 GE and repeated exposure (for 3 consecutive days) was essential for transmission via the fecal-oral route, and this was successful in only one of three pigs in the present study.

Others have reported that HEV infection in nonhuman primates is dose dependent (20, 39). All three shedder pigs in this present study shed what is considered a high titer of HEV (2.7 X 10^6 to 13.2 X 10^6 virus genomic copies per g of feces) in their feces, particularly during the third day of the exposure inoculation (11 dpi). Feces from all three positive control pigs were pooled prior to inoculation. However, only one pig in the fecal-oral exposure group became infected. The reason for the discrepancy in HEV transmission among three pigs in the fecal-oral exposure group remains obscure.

HEV is a temperature-sensitive, labile virus (4). The infectivity of HEV is reportedly diminished after a rapid change in temperature (4). In this study, the length of time between the collection of fresh feces from the rectum of HEV-infected pigs and the exposure inoculation was less than 5 minutes. The pooled fresh feces collected were at ambient

temperature until subsequently given orally to naïve pigs. Inactivation of the virus in such a short period under these conditions should be minimal.

In contrast to the fresh feces used in this experiment, a suspension (10% [wt/vol] in PBS) of feces was used as inocula in our previous study in which we failed to transmit HEV by the fecal-oral route (17). In that study, the fecal suspensions were clarified by centrifugation at 1,100 X g for 10 min, and the supernatant was stored frozen at -80^0 C for up to 4 years (17). The same frozen fecal suspension was used as the intravenous inocula for the "shedder" pigs in the current and previous experiments (13, 17, 18). The freeze-thaw process might have affected the ratio of infectious to defective HEV particles (4, 23) and contributed to the unsuccessful transmission of the HEV via the oral route in a previous experiment (17).

Proteolytic degradation of the infectious HEV particles in the intestinal tract of the host may also inactivate infectious HEV particles (4). However, inactivation of the HEV particles within the intestinal tract may be offset by newly assembled HEV virions originating from *in situ* replication in the intestines, thus maintaining a high amount of the infectious virus particles in the feces (42). HEV infection in the intestinal mucosa without a systemic HEV infection may explain why one positive-control pig and some contact control pigs shed HEV in feces but did not develop HEV viremia (one of three pigs) or anti-HEV antibodies (two of three pigs). The lack of systemic HEV infection may explain the inability to detect HEV viremia and the insufficient serum antibody response in some pigs.

HEV RNA was not detected in the tonsil and nasal cavity secretions tested in this experiment during the period of viremia. There is no evidence that tonsils and salivary glands support replication of swine HEV (42), so it is not surprising that the pigs in the tonsil and nasal exposure group failed to become infected. This is further supported by the lack of

evidence of transmission to noncontact pigs breathing the same air as the pigs in the fecal-oral exposure group. It is unlikely that the nasal mucosa or respiratory tracts are the portals of viral shedding or routes of HEV infection. Urine is also less likely to be a source of HEV transmission in pigs, since there is no evidence that swine HEV replicates in kidney (42).

Repeated use of the same needle and syringe is common when vaccines and drugs are administered to growing pigs on commercial pig farms. We chose to use a *Mycoplasma hyopneumoniae* vaccine since this type of vaccine is commonly administered to pigs between 5 to 12 weeks of age, when HEV transmission likely occurs (24). The volume of residual blood in used needles varies depending on the route of injection, the size of the hypodermic needle, and the volume and type of syringe. Up to 7.5 μl of residual blood was transferred during sharing of needles by intravenous drug users. In needle sharing simulations, the blood volume was increased when a 2-ml syringe was used than when a 1-ml syringe was used (12). In the present study, we used an 18-gauge and 1-in.-long needle on a 3-ml syringe for three consecutive days of the exposure inoculation process. Much larger multiple-dose syringes (i.e., 50 ml and larger) are typically used in the swine industry. The doses were administered into muscle in the present study, so residual blood in the needle would be less than that of the needles used for intravenous injection. Furthermore, there is no evidence that skeletal muscle is the site of replication for HEV (42) or that pork meat contains a detectable amount of HEV (17).

The HEV viremic period is variable and transient and generally lasts only about 1 to 2 weeks (13, 16, 25). A recent survey of 99 pigs from several regions in Indonesia reported a sharp contrast between the prevalence of anti-HEV seroconversion (72%) and the number of pigs whose serum samples tested positive for HEV RNA (1%) (41). The transient and/or

intermittent characteristic of HEV viremia may contribute in part to the ineffective transmission of HEV through contaminated needles. From a comparative perspective, the injection of therapeutic drugs was not a risk factor for the high prevalence of anti-HEV antibodies in Danish patients who may have contracted HEV infection via the blood-borne route (6). Swine veterinarians in the United States who reported experiencing needle sticks or cuts with blood-to-blood contact were not at a significantly higher risk of anti-HEV seropositivity compared to those who had no history of such exposure (28). The inability to transmit HEV infection with used needle exposure in this experiment and the lack of epidemiological evidence of transmission of swine HEV to humans with exposure to pig blood may be due to an inadequate amount of infectious HEV particles in the blood on the contaminated needle or necropsy knives.

In summary, the findings of the present study indicate that experimental fecal-oral transmission of swine HEV in pigs did occur but was not efficient. Efficient transmission of swine HEV in pigs via the fecal-oral route may require repeated exposure and high doses. Evidence of the transmission of swine HEV through tonsil and nasal secretions was lacking. It also appears unlikely that the repeated use of needles represents a common means of swine HEV transmission in pigs. The ubiquitous nature of swine HEV in the swine population and the presence of swine HEV in pig feces for a considerably longer period than the length of HEV viremia also favors the fecal-oral route as the primary route of swine HEV transmission. However, it remains unknown if there are undetermined factors that may facilitate swine HEV transmission through the fecal-oral route to the degree that HEV infection is reportedly to be widespread in global pig populations.

Acknowledgments

We thank Robert Purcell and Suzanne Emerson for providing the human HEV Sar-55 antigen and the swine HEV stock. This work was supported by a grant (to P.G.H.) from the Iowa Livestock Health Advisory Council and in part by grants (to X.-J.M.) from the National Institutes of Health (AI01653 and AI46505).

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Table 1. Viremia, fecal shedding, and seroconversion in positive-control (shedder) pigs inoculated intravenously with a swine HEV infectious stock

Pig	Swine HEV parameter	Swine HEV parameter status[a] on the following dpi:								
		0	7	14	21	28	35	42	49	56
A	Fecal shedding	-	+	+	+	-	-	-	-	-
	Viremia	-	-	-	-	-	-	-	-	-
	Anti-HEV	-	NT[a]	NT	NT	-	NT	NT	NT	-
B	Fecal shedding	-	+	+	+	+	-	-	-	-
	Viremia	-	-[b]	+	-	-	-	-	-	-
	Anti-HEV	-	NT	NT	NT	-	NT	NT	NT	+
C	Fecal shedding	-	+	+	+	-	-	-	-	-
	Viremia	-	-	+	+	-	-	-	-	-
	Anti-HEV	-	NT	NT	NT	+	NT	NT	NT	+

[a] -, negative; +, positive; NT, not tested.

[b] Pig B was negative for HEV RNA at 7 DPI but positive on 5, 6, and 8 to 14 dpi.

Table 2. HEV genomic copy number in fecal and serum samples of positive-control pigs collected during the days when samples were taken from them for inoculation and exposure of naïve pigs

Shedder pig	Sample	HEV genomic copy no.[a] on the following dpi: 9	10	11
A	Feces	-[b]	2.7	10.5
A	Serum	-	-	-
A	Tonsil and nasal swab	-	-	-
B	Feces	2.9	3.7	10.5
B	Serum	2.8	4.4	6.6
B	Tonsil and nasal swab	-	-	-
C	Feces	3.8	6.2	13.2
C	Serum	-	-	-
C	Tonsil and nasal swab	-	-	-

[a] The number of virus genomic copies X 10^6 per gram of feces or per milliliter of serum is shown for the feces and serum samples, respectively.
[b] The sample was negative for HEV RNA by RT-PCR, so quantitative PCR was not done.

Table 3. Detection of anti-HEV serum antibodies by ELISA and of HEV RNA by RT-PCR in pigs inoculated with biological samples from HEV-infected pigs

Group	Inoculation Route	No. of positive pigs ($n = 3$)[a]		
		Seroconversion	HEV RNA in feces	HEV RNA in serum
1	Swine HEV positive control[b]	2/3	3/3	2/3
2	Direct contact exposure to positive control	2/3	3/3	1/3
3	Tonsil and nasal secretions exposure	0/3	0/3	0/3
4	Needle exposure	0/3	0/3	0/3
5	Fecal-oral exposure	1/3	1/3	0/3
6	Negative control	0/3	0/3	0/3
7	Sham tonsil and nasal secretions exposure	0/3	0/3	0/3
8	Sham needle exposure	0/3	0/3	0/3
9	Sham fecal-oral exposure	0/3	0/3	0/3

[a] Number of positive pigs of three pigs tested on 56 dpi.
[b] Inoculated intravenously with swine HEV.

CHAPTER 6.

HEPATITIS E VIRUS IN PIG MANURE STORAGE FACILITIES IS INFECTIOUS TO PIGS BUT EVIDENCE OF GROUND WATER CONTAMINATION IS LACKING

A paper submitted to

Applied and Environmental Microbiology

Chaiyan Kasorndorkbua, Fang F. Huang, Denis K. Guenette,

Pete J. Thomas, Xiang-Jin Meng, Patrick G. Halbur

Abstract

Fresh feces, manure (from earthen lagoons and/or concrete pits), and drinking and surface water samples were collected in the summer and autumn of 2002 from 28 pig farms in the Midwestern United States. All samples were tested for swine HEV RNA by RT-PCR assay. Seven of 28 farms had fresh fecal samples that contained HEV RNA. In pit samples, 15 of 28 farms contained HEV RNA. Of 8 farms that had lagoons, 3 contained HEV RNA. The highest virus titers were 10 and 10^3 genome equivalent per 60 ml of manure in lagoon (designated as lagoon A) and pit (designated as pit B) samples, respectively. None of the water samples were positive for HEV RNA. To determine the infectivity of the HEV found in the positive lagoon or pit samples, pigs were inoculated either intravenously (n=3) or

orally (n=3) with the lagoon A or pit B manure. Four pigs inoculated intravenously with swine HEV served as positive controls. Three pigs served as uninoculated controls. All positive control pigs became infected and shed the virus in feces, and 3 of 4 developed anti-HEV antibodies. Two pigs in the intravenously-inoculated pit B group shed the virus in feces and one of the pigs seroconverted to anti-HEV antibodies. None of the pigs in the negative control, the lagoon A oral, the lagoon A intravenous, or the pit B oral group shed the virus in feces. The findings indicate that HEV found in pig manure was infectious to pigs when inoculated intravenously. Pit manure is a potential source of infection of pigs and for contamination of the environment with swine HEV. However, contamination of drinking or surface water with HEV was not found on or nearby the pig farms.

Introduction

Hepatitis E virus (HEV) is the etiologic agent of an acute self-limiting non-A, non-B hepatitis in humans (46). The virus is a non-enveloped positive–sense single-stranded RNA virus and is recently given taxonomic status in the genus of *Hepevirus*, a member of the family *Hepeviridae* (10). Human infection with HEV typically manifests as acute icteric hepatitis. However, chronic hepatitis associated with HEV has not been reported (11, 28, 46). The overall mortality is low, except for pregnant women that may suffer severe and fatal hepatitis with mortality rates up to 25% (28, 29, 30). HEV infection is distributed worldwide and is endemic in parts of Africa and Asia (28, 50).

Human HEV outbreaks in endemic regions are reportedly associated with flooding and heavy rains through the fecal-oral route of transmission (8, 11). HEV infection in non-endemic industrialized countries is sporadic and is often reported in human patients with a

history of traveling to endemic countries; however, increasing reports of sporadic human HEV infection in non-endemic countries such as Japan, Greece, Italy, and the United States (U.S.) in patients without a history of traveling to endemic regions have been reported recently. The human HEV discovered in non-endemic, industrialized countries appears to be closely related to the swine HEV found in the same countries (5, 11, 43, 45, 47, 48, 61).

Pigs are a natural host for HEV (22, 35, 38, 61). HEV infection is globally ubiquitous in the pig population (6, 13, 19, 22, 38, 40, 41, 42, 47, 50, 51, 54, 55, 59, 60, 63). The fecal-oral route is thought to be the primary mode of HEV transmission (11, 60). Pigs infected with HEV shed the virus in feces in large amounts for 3-4 weeks whereas viremia is transient and persists for 1 to 2 weeks (15, 25, 26). A study of HEV infection incidence in pig farms suggested that swine HEV is likely transmitted via the fecal-oral route (60). Current pig raising practice allow repeated exposure of pigs to feces and thus potentially to high doses of HEV (26, 60).

Evidence of animal-to-human transmission of HEV has recently been documented in a case where ingestion of uncooked liver obtained from a wild boar may have transmitted the infection (32). Consumption of uncooked meat collected from wild deer has caused human hepatitis E infection in 2 Japanese families (52). Livers collected from pigs experimentally inoculated with swine HEV contained viable HEV and the virus in the livers was infectious to pigs when inoculated intravenously (26).

Human and animal raw sewage has been reported to contain HEV and the virus found in the human sewage was infectious as confirmed by a bioassay in non-human primates inoculated intravenously (43, 44). Pig-to-human transmission of HEV has been suggested by serological surveys (9, 34, 35, 58). It has been shown experimentally that a U.S. human HEV

strain infects pigs and swine HEV infects non-human primates (15, 37). Food-borne or blood transfusion routes have been implicated as likely means of origin of sporadic cases of human HEV infection in non-endemic industrialized countries (31, 32, 52, 62). Contamination of drinking or surface water in the vicinity of pig production facilities is possible and of concern (50). The status of HEV in pig manure in storage facilities such as concrete pits and earthen lagoons remains to be investigated. If HEV is indeed present in stored pig manure it needs to be determined if the virus is infectious to pigs or other animals. The objectives of the present study were; (i) to determine whether HEV is present in pig feces and stored manure, on-site drinking water supplies, and surface water near swine production facilities, and (ii) to determine if HEV found in pig manure and/or water samples is infectious to naïve pigs.

Materials and Methods

Fecal sample collection and processing. In the late summer and autumn of 2002, 28 pig farms located in the Midwestern United States (Iowa) were visited and sampled. Fresh feces from 5 live pigs per farm were collected, pooled, and prepared as 10% (wt/vol) fecal suspension in DEPC water-based phosphate-buffered saline (PBS).

Manure sample collection and processing. Samples of manure in pits and lagoons were randomly obtained from 10 different sites of each manure storage facility and collected in a 50 ml sterile centrifuge tube. Ten manure samples were collected from the concrete pit under the slats of each finishing facility. The samples were taken from 10 randomly selected sites in each pit. Each sample was collected from a depth between one and two feet below the surface and directly below the pig pens. For the lagoon manure samples, 10 samples were randomly collected at a depth of 4 to 6 feet under the surface crust.

All samples were transported on ice to the laboratory. The ten tubes of each manure sample from each storage facility (pit and/or lagoon) were pooled together in two aliquots of 250 ml. All fecal suspensions and manure samples were kept at -80° C until tested by RT-PCR assay. Portions (60 ml) of the manure samples were subjected to ultracentrifuge protocols to concentrate virus particles (44). The manure samples were clarified at 1,100 x g for 10 min at 4° C. The supernatant was ultra-centrifuged at 229,600 x g for 1 h at 4° C to pellet the virus particles. The virus pellet was then diluted with 10 ml of 0.25 M glycine buffer (pH 9.4) on ice for 30 min. The dissolved virus pellet was re-centrifuged at 12,000 x g for 15 min at 4° C to discard solid materials. The supernatant of the virus pellet was saved and ultra-centrifuged at 229,600 x g for 1 h at 4° C to produce a final virus concentrate. The final virus concentrate was resuspended with 400 µl of DEPC water-based PBS. Two aliquots of 200 µl each were prepared for qualitative nested RT-PCR and for semi-quantitative nested RT-PCR which were kept at -80° C until tested. The manure concentrate samples that were positive for HEV RNA by the qualitative RT-PCR were further titered for HEV by the semi-quantitative RT-PCR.

Water sample collection and processing. On each farm visited, 10 liters of on-site drinking water and 10 liters of water from the nearest upstream and downstream surface water (creeks, streams, or rivers) within a 0.25 to 6 mile radius of the pig farms were collected and concentrated. All water samples were collected in a sterile 10-liter container. Drinking water samples were directly obtained from a hydrant or faucet in or near the pig barns. Surface water samples were directly collected from the water source by submerging the container below the water surface. The water samples were transported in a cooler with

ice packs to the laboratory and were processed for virus concentration upon arrival within a day of collection.

The concentration protocols were modified from Abbaszadegan et al., 1999 (1) and the guidelines for detection of enteric viruses recommended by U.S. Environmental Protection Agency, 1995 (4). Ten liters of water samples were run through a sterile unit of a filter housing (CUNO, Inc., Meriden, CT.) and cartridge (Virosorb® MDS1, CUNO, Inc., Meriden, CT.). All flow-through water was discarded. Once the filtering process was complete, 1 liter of autoclaved 0.25 M glycine buffer (pH 9.4) was used to elute the entrapped virus particles in the filter cartridge. The elution process was performed twice by using the first viral eluate for the second elution process. The virus eluate was immediately neutralized at pH 7-7.4 with 1 N HCL (pH 1.5), mixed vigorously, and kept on ice before proceeding to the virus concentration process by organic flocculation (4). The neutralized virus eluate was adjusted to lower its pH to 3.5 with 1 N HCL and 12 g of autoclaved beef extract powder and stirred vigorously for 15 min. The pH-adjusted virus eluate was then centrifuged at 3,000 x g for 10 min at 4° C. The supernatant of the virus eluate was discarded and the resulting pellet was re-suspended with 50 ml of DEPC water-based PBS. The suspended virus pellet was made into 2 aliquots of 25 ml. One aliquot was subjected to the centrifugal concentration process to adjust the eluate volume for qualitative RT-PCR testing and the other was kept at -80° C for an archival purpose. The virus eluate for RT-PCR was subjected to 1,100 x g for 20 min in a Centrifugal Filter unit (Centricon®, Millipore Corporation, Billerica, MA.) to concentrate the eluate volume for the RNA extraction steps.

To determine the sensitivity of the water concentration used in the present study, 10 liters of tap water was spiked with one ml of a swine HEV infectious fecal pool (10% wt/vol)

with a titer of 10^2 genome equivalent (GE) per ml (37). The swine HEV-spiked water was processed through the protocols described above and the spiked virus eluate was tested by swine HEV-specific RT-PCR assay and found to be positive for swine HEV RNA.

Universal nested RT-PCR. Hepatitis E virus is heterogenic in the United States swine populations (22). A nested RT-PCR assay designed to detect genetically-variable strains of HEV was used to amplify HEV nucleic acid (22). This universal RT-PCR was used to test the concentrated manure (collected either from concrete pit or earthen lagoon facilities) and concentrated water samples (collected either from surface water sources or on-site drinking water). The modified spin column method (QIAamp; Qiagen, Chatsworth, CA.) was used to extract total RNA from 140 µl of concentrated manure and water samples or fecal suspensions (3). The RNA extract (11.5 µl) was then immediately used for the reverse transcription reaction for HEV cDNA synthesis. The first round PCR primers comprised the forward primer F1 [5'-AATTATGCC(T)CAGTAC(T)CGG(A)GTTG-3'] and the reverse primer R1 [5'-CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC-3']. The second round primers were the second forward primer F2 [5'-GTT(A)ATGCTT(C)TGCATA(T)CATGGCT-3'] and the second reverse primer R2 [5'-AGCCGACGAAATCAATTCTGTC-3']. The second-round amplified PCR product of 348 base pairs (bp) in length was visualized by gel electrophoresis. For the PCR parameters used in the universal RT-PCR reactions, we strictly followed the protocol published by Huang et al., 2002 (22). To avoid false positive results from contamination during PCR reactions, positive (a standard infectious fecal pool containing swine HEV) and negative controls (sterile PBS) were added in every set of PCR reactions.

Semi-quantitative nested RT-PCR for titration of HEV in manure or water samples. To determine the virus titer in samples tested positive for HEV RNA, serial 10-fold dilution in DEPC water-based PBS, ranging from 10^{-1} to 10^{-6} dilution was made and subjected to the universal RT-PCR. The virus titer was reported as genome equivalent (GE) at the highest dilution found positive by the RT-PCR (36, 37).

HEV genomic sequencing and phylogenetic analysis. PCR products amplified from pooled feces and manure were purified using the glass milk procedure with a GENECLEAN kit (Bio 101 Inc., Carlsbad, CA.) and then directly sequenced at the Virginia Tech DNA Sequencing Facility. Sequences of the PCR products were determined for both DNA strands. The primer sequences of the universal RT-PCR were excluded from the sequence and phylogenetic analysis. The resulting partial 225-bp sequences of positive feces and manure were analyzed and compared with the corresponding regions of prototype swine HEV and some known strains of swine HEV available in the GeneBank database by the MacVector computer program (Oxford Molecular Inc., Campbell, CA.). Phylogenetic analysis was conducted with the aid of the PAUP program (David Swofford, Smithsonian Institute, Washington, D.C., distributed by Sinauer Associate Inc. Sunderland, MA.). A heuristic search with 1,000 replicates was used to produce a phylogenetic tree. The geographic origins and the GeneBank accession numbers of the nucleotide sequences of the HEV strains, representing four major genotypes (11) and including the recently-characterized avian HEV (16, 21), used in the phylogenetic and sequence analyses are as follows: human (h) HEV-Sar-55 (Pakistan, M80851, genotype 1), hHEV-Mexican (Mexico, M74506, genotype 2), the prototype swine HEV (USA, AF082843, genotype 3) and hHEV-U.S.-2 (USA, AF060669,

genotype 3), hHEV-T1 (China, AJ272108, genotype 4), and avian (a) HEV (USA, AY043166).

Virus inocula. The swine HEV inocula used for inoculation of the positive control pigs was prepared from an infectious stock of feces collected from pigs intravenously infected with the prototype U.S. strain of swine HEV (38). The inocula contained a HEV titer of $10^{4.5}$ 50% pig infectious dose (PID_{50}) per ml, approximately equivalent to 10^6 GE per ml (37). The inocula were kept at -80° C until used.

Swine bioassay. To determine if HEV RNA found in manure samples represented infectious virus particles, the HEV-positive pit and lagoon samples with the highest titer of HEV measured by semi-quantitative RT-PCR was prepared as inocula for naïve pigs in a swine bioassay. Nineteen, 6-week-old, specific-pathogen-free pigs were randomly separated into 6 groups of 3-4 pigs. All pigs were anti-HEV seronegative by ELISA and free of HEV in feces by swine HEV-specific nested RT-PCR. Three pigs served as uninoculated controls. Four positive-control pigs were inoculated intravenously with the prototype U.S. swine strain (37). Four groups of three pigs each were inoculated with 25 ml of either a HEV-positive pit or lagoon sample via intravenous or oral route. The selected lagoon sample was collected from farm #19 and designated as lagoon A and the selected pit sample from farm #12 and designated as pit B.

For preparation of the inocula, portions (150 ml) of the manure collected from lagoon A or pit B was dissolved in sterile DEPC water-based PBS to produce a manure suspension. The suspensions were clarified at 1,100 x g for 10 min at 4^0 C. Each pig was given 25 ml of the supernatant by intravenous inoculation (IV). The intravenous inoculation was performed for 5 consecutive days using 5 ml of the inocula per pig per day. The accumulative HEV titer

each pig received by intravenous inoculation was approximately 10 and 10^3 GE for lagoon A and pit B, respectively. For oral inoculation, 25 ml of pooled pig manure from lagoon A or pit B was given to naïve pigs all at one time by oral gavage. The HEV titer each pig received for oral inoculation was approximately 10 and 10^3 GE for lagoon A and pit B, respectively. Serum samples and fecal swabs were collected weekly for anti-HEV serum antibodies and detection of HEV RNA. The bioassay was terminated on 56 days postinoculation (dpi).

Detection of anti-HEV IgG antibodies by ELISA. Serum samples were tested by an enzyme-linked immunosorbent assay (ELISA) using a purified 55-kDa truncated recombinant putative capsid protein of human HEV Sar-55 strain as the antigen (37, 38). Each serum sample was tested in duplicate, and mean optical density values above 0.3 were regarded as positive samples. Preimmune and hyperimmune sera were used as negative and positive controls, respectively. All pigs used in the swine bioassay were negative for anti-HEV immunoglobulin (Ig) G antibodies prior to inoculation.

Swine HEV-specific nested RT-PCR for fecal samples collected from the swine bioassay. Since the prototype U.S. swine HEV was used as the inocula for positive controls, a nested RT-PCR assay designed to specifically detect the prototype U.S. strain of swine HEV (15) was used to amplify HEV RNA in feces collected from the bioassay pigs in order to compare and differentiate between the HEV genomic sequence amplified from the prototype swine HEV (collected from the positive control pigs) and the genetically-variant HEVs potentially found in the lagoon or pit inocula (collected from pigs inoculated with the lagoon or pit inocula). The RNA extraction method and the volume of fecal suspensions used for this swine HEV-specific RT-PCR were similar to those performed for the universal RT-PCR. However, the primers for the swine HEV-specific RT-PCR and PCR parameters were

different from those used for the universal RT-PCR as follows (15): the first round PCR primers comprised the forward primer F1 (5′-AGCTCCTGTACCTGATGTTGACTC-3′) and the reverse primer R1 (5′-CTACAGAGCGCCAGCCTTGATTGC-3′). The second-round PCR primers comprised the forward primer F2 (5′-GCTCACGTCATCTGTCGCTGCTGG-3′) and the reverse primer R2 (5′-GGGCTGAACCAAAATCCTGACATC-3′) and produced an amplified PCR product size of 266 bp. To synthesize HEV cDNA, portions (11.5 μl) of RNA extract was amplified in a reverse transcription reaction with R1 reverse primer and Superscript II reverse transcriptase (Invitrogen) at 42° C for 1 h. The cDNA (10 μl) was used as template in a 100-μl PCR reaction. The first-round PCR reaction was initiated with the activation of ampli*Taq* Gold DNA polymerase (Applied Biosystems) at 95° C for 9 min, followed by repeated 39 cycles of denaturation, annealing, and extension as follow; 94° C for 1 min, 52 ° C for 1 min, and 72 ° C for 1.5 min, respectively, and finally, an incubation at 72 ° C for 7 min. Similar PCR parameters were applied to the second round PCR reaction. The amplified PCR products were inspected on gel electrophoresis.

Results

Detection of HEV in feces, manure, and water. HEV RNA was detected in pooled fresh feces from 7 of the 28 farms. Fifteen of 28 farms were found to have HEV-positive manure samples collected from concrete storage pits. Three of 8 lagoons were positive for HEV RNA (Table 1). None of the concentrated water samples collected from on-site drinking water supplies or the nearest surface water sources (creeks, streams, or rivers) were positive for HEV RNA (data not shown). The highest virus titer in manure samples that

tested positive for HEV RNA was approximately 10 and 10^3 GE in lagoon A and pit B, respectively (Table 1).

Determination of the infectivity of HEV found in manure samples. The swine bioassay results are summarized in Table 2. All positive-control pigs shed HEV in feces from 14 to 49 dpi and 3 of 4 positive control pigs developed anti-HEV antibodies by 56 dpi. Uninoculated control pigs remained HEV-free in feces and negative for anti-HEV IgG antibodies by the termination of the study on 56 dpi. Two pigs inoculated intravenously with the pit B inocula became infected with swine HEV. The presence of HEV RNA in feces was detected in one pig in the pit B inoculated group by 14 dpi and in the other pig by 21 dpi. Both pigs continued to shed HEV in feces through 28 dpi. The pig that shed HEV in feces from 14 to 28 dpi seroconverted to HEV, whereas the other pig remained seronegative on termination of the study at 56 dpi. One of 3 pigs inoculated intravenously with manure from lagoon A seroconverted to anti-HEV antibodies by 56 dpi.

Genetic variation of HEV isolates in pooled feces. Of 7 pooled fecal samples positive for HEV RNA, nucleotide sequence analyses of the amplicons revealed that the 7 HEV isolates shared 88-94% identity with each other and 88-96% identity with the prototype U.S. swine HEV (Table 3).

Genetic variation of HEV isolates in the pit or lagoon manure samples. A phylogenetic tree was built based on 225 bp-length sequences. Phylogenetic analysis indicates that HEVs amplified from manure samples collected from either pit or lagoon facilities are segregated within genotype 3 which is where the prototype swine HEV and the US-2 strain of human HEV are clustered (Figure 1). There were two farms (farm #18 and # 19) where the HEVs found in pig feces and the pit or lagoon manure samples were more

closely related to each other than to the HEVs from different farms. On one farm (F18 and P18), the HEVs from feces and pit manure were identical (Figure 1).

Swine bioassay. The amplicon of HEV RNA from feces collected at 21 dpi from a pig (designated as pig I in Table 4) inoculated intravenously with pit B was 100 % identical to the amplicon of HEV RNA from the pit B intravenous inocula that the pig received. The amplicon of HEV RNA from feces collected on 21 dpi from the other pig (designated as pig II) in the pit B intravenous group was 93% identical to the pit B intravenous inocula that the pigs received (Table 4).

Discussion

Pig manure may be an appropriate sample, perhaps even better than fresh feces, to confirm the status of HEV infection of a population of pigs. More HEV positive sites were detected by testing manure samples from pits compared to testing fresh feces. The concentration protocols used in this study for detection of HEV in manure offer a reliable tool to detect small amounts of HEV RNA in large volumes of manure. The manure in the pits and lagoons likely represents HEVs from many pigs present at one time or another on that site over an extended period of time.

In the present study, all manure samples were collected during late summer and autumn months of a year when the ambient temperature would be quite high compared to that in the winter months in the Midwestern U.S. The high temperature range at the time the manure samples were collected could have enhanced the biodegradation of organic waste in the manure storage facilities and decreased the amounts of HEV nucleic acids detectable by

RT-PCR and the viability of the virus assayed. In contrast, low temperature during winter months may preserve the infectivity of environmental viruses for longer periods (39).

The HEV isolates from the pooled feces and manure were genetically diverse but all clustered in the genotype 3 with the prototype U.S. swine HEV. Nucleotide sequence comparison showed that HEVs isolated from the pooled feces and pit and/or lagoon samples of the same farm are genetically more closely related to each other than those from different farms.

Wild rats likely reside on pig farms. Therefore it is also possible that rats are an alternative source for contamination of pig manure storage facilities since a high prevalence of anti-HEV antibodies has been reported in wild rats (12, 17, 18, 23). Demonstration of high affinity of partially amplified HEV genomes between wild rats and humans in Nepal suggests that wild rats may be a reservoir for HEV infection in humans and play a major role in HEV epidemics in such HEV-endemic countries (17). Interestingly, all HEVs found in the manure samples tested in the present study are phylogenetically clustered in the genotype 3, which is genetically distant from the rat HEV reported in Nepal, which was clustered in the genotype 1 (17).

HEVs are genetically heterogeneic and both human and swine strains of HEV tend to be genetically clustered according to their geographical origins (6, 7, 19, 22, 40, 43, 49, 51, 54, 61). When genomic characterization of rat HEV becomes available a phylogenetic comparison with HEVs from humans, pigs and the manure samples in the current study will provide further insights into the likelihood that wild rats are a natural reservoir for HEV transmission to pigs and perhaps humans as has been suggested by serological studies (12, 17, 18, 23). Wild deer may also gain access to manure in lagoons in the Midwestern U.S.

Since evidence of food-borne zoonotic transmission of HEV from deer to humans has been reported in Japan (52) it is tempting to speculate that wild deer may be another reservoir for HEV. To date, the status of anti-HEV antibodies in wild deer has not been reported in the U.S. It remains to be determined whether HEV exists in the U.S. wild deer populations and if deer may contribute to contamination of the environment with HEV and HEV transmission among humans, pigs, and other animals.

The swine bioassay indicates that HEV found in pig manure was infectious to naïve pigs when inoculated intravenously. Partial nucleotide sequences of the PCR amplicon amplified from the pit B inocula and 1 pig (pig I in Table 4) inoculated intravenously with the pit B inocula were identical, and were different from the amplicon amplified from the positive control pigs inoculated intravenously with the prototype swine HEV. This confirms that the virus found in the pit B inocula was infectious and replicated in pig I and was subsequently shed in the feces of that pig. The lack of complete nucleotide identity between the virus found in feces from pig II and the pit B inocula (and pig I) may reflect the genetic variability of different HEVs (HEV quasispecies) found in the manure since the amplicon from feces collected from pig II was different from that in the pit B manure or the prototype swine HEV. Evidence for quasispecies of HEV has recently been reported in an epidemic of human HEV infection (14). As an RNA virus, HEV may be prone to replication errors due to the function of the non-proofreading polymerase enzyme, leading to diverse populations (14). Another possibility that could have contributed to such a difference in the nucleotide identity includes random mutations during the PCR reaction (27).

The swine bioassay findings are in accordance with a report of analysis in raw sewage drained from an urban area in Spain where HEV present in the biological waste facilities

remained infectious to experimentally-inoculated rhesus monkeys (44). Subsequently, the authors reported the presence of HEV in raw sewage drained from pig slaughterhouses. However, an animal bioassay was not performed to determine the infectivity of the HEV found in the raw pig sewage (43). HEV has recently been found in raw human sewage collected from industrialized countries including the U.S. According to the partial nucleotide sequence analysis, the HEV found in the human sewage was more homologous to the prototype U.S. swine HEV than to the US-1 and US-2 strains of human HEV (98.4% versus 91.0%) (7). The prototype swine HEV is able to infect non-human primates (37).

Although most individuals infected with swine HEV are likely subclinically infected (9, 34, 35), it is important to understand the source of such infections. Possibilities include consumption of pork products or consumption of water or food contaminated with pig feces. It is also possible that human sewage facilities are opened to wild rats or other animal reservoir species that shed HEV and that viruses detected in those facilities are not from humans.

One pig each of the positive control and the pit B intravenously-inoculated (pig I) group remained HEV seronegative at the end of the study (Table 2.). It has been previously demonstrated that a few pigs experimentally-inoculated intravenously with swine HEV may have a delayed response to the infection and seroconversion to anti-HEV antibodies may occur beyond the 56 day duration of the experiment (26, 38). A recent study using an infectious clone of swine HEV in pigs showed that one pig inoculated intra-hepatically with a low virulent HEV clone did not develop detectable anti-HEV antibodies by 56 dpi (20).

One pig inoculated intravenously with the lagoon A inocula seroconverted to HEV by 56 dpi without detectable shedding of the virus in feces (Table 2). A possible explanation for

this discrepancy may be that the virus in the lagoon A intravenous inocula was able to induce an immune response in that particular pig (33); however, as the swine bioassay results indicate, the virus titer of 10 GE failed to induce HEV infection. Dose-dependent infectivity of HEV has been suggested in pigs (37) and nonhuman primates (53). It has been demonstrated that the effective titer to induce HEV infection, replication, and virus shedding in feces in pigs was 10^2 GE when inoculated intravenously (26). An alternative explanation is that the pig did become infected and had brief localized replication of HEV in the intestines (57) and viremia and virus shedding in feces may have been intermittent and went undetected by weekly RT-PCR testing.

Experimental studies of swine HEV transmission in pigs showed that fecal-oral transmission of HEV in pigs may occur, however it likely requires a higher dose and repeated exposure for successful transmission (24, 26). The results of this study are consistent with the previous findings that experimental oral inoculation is less efficient and requires a higher virus load in induction of HEV infection than intravenous inoculation since none of the three pigs inoculated orally with the pit B inocula became infected, whereas 2 of 3 pigs inoculated intravenously did (2, 11, 26).

HEV was not found in the ground or surface water samples tested with the concentration methods used in the present study. However, the methods used in the current study were able to detect HEV at a titer of 10^2 GE when water was spiked with the prototype swine HEV. The dilution of the concentrated water samples with sterile PBS and the extraction protocols using the microspin column technique have been reported to minimize the inhibition of PCR reaction by potential inhibitors that have contaminated samples prior to the PCR reaction (3, 56). It is, therefore, possible that either the water samples tested in the

study lacked HEV particles or the level of contamination by HEV was below the detection ability of the concentration methods applied in the study.

The RT-PCR used in the study has proven to be useful for the detection of swine HEV in animal organic waste reported here and raw sewage and biological samples reported elsewhere (7, 22, 26, 43, 44). However, the RT-PCR assay does not differentiate between infectious and non-infectious virus particles since it is designed to amplify targeted nucleic acid of HEV genomes in the samples which may not reflect virus viability (1). Even though the bioassay is costly, until an effective cell culture system that supports in vitro HEV replication is developed, the swine bioassay may be essential to determine the infectivity of HEV found in the pig manure. It seems unlikely that consumption of water with levels of HEV below the level of detection with these methods would result in HEV infection in pigs.

Cross species transmission of swine HEV to non-human primates has been demonstrated (37). Thus, it is prudent to assume that HEV present in pig manure waste could remain infectious and pose a risk as a source of transmission of HEV to humans. It is currently unknown how long HEV in pig manure waste can survive and remain infectious. More research on the survivability of HEV in different manure storage facilities and under different management practices of manure storage and application are warranted.

In summary, HEV is present in pig farms and manure collected from concrete pits or earthen lagoons. Pig manure may be an appropriate sample, probably more suitable than fresh pig feces, to determine the presence of HEV on pig production sites. The swine bioassay findings indicate that HEV found in pig manure is infectious to naïve pigs when inoculated intravenously. The presence of infectious HEV in manure storage facilities suggests that pit manure is a potential source of infection for pigs, other animal species,

humans, and for contamination of the environment with swine HEV. However, evidence of contamination of drinking water or surface water with HEV was not found on or nearby the pig farms with the detection methods used in the present study.

Acknowledgments

The authors thank Drs. Robert H. Purcell and Suzanne U. Emerson of the National Institutes of Health, Bethesda, Maryland for providing the Sar-55 antigen and the swine HEV inocula. We are grateful to Drs. Dennis Winter and Doug Quam for access to pig farms. The study was supported by a grant from the National Pork Board Pork Checkoff Dollars (USA).

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Table 1. Detection of HEV RNA in pooled pig feces and environmental samples collected from 28 pig farms.

Farm ID	Type of Farm	Feces	Pit (Virus titer [a])	Lagoon (Virus titer)
1	Farrow to finish	- [b]	+ (10^3)	NA [c]
2	Farrow	-	-	-
3	Farrow to finish	-	-	-
4	Farrow to finish	-	-	NA
5	Grow/finish	-	+ (10)	NA
6	Grow/finish	-	NA	-
7	Grow/finish	-	+ (10)	NA
8	Grow/finish	-	-	NA
9	Grow/finish	+	+ (10)	NA
10	Wean to finish	-	-	+ (10)
11	Farrow to finish	-	+ (10)	NA
12	Grow/finish	+	+ (10^3)	NA
13	Farrow to finish	-	+ (10)	NA
14	Grow/finish	-	+ (10)	NA
15	Grow/finish	-	+ (10^2)	NA
16	Grow/finish	-	+ (10)	NA
17	Farrow to finish	-	NA	+ (10)
18	Wean to finish	+	+ (10)	NA
19	Farrow to finish	+	NA	+ (10)
20	Wean to finish	-	-	NA
21	Farrow to finish	+	+ (10)	NA
22	Grow/finish	+	+ (10)	NA
23	Farrow to finish	-	NA	NA
24	Grow/finish	-	NA	NA
25	Grow/finish	+	+ (10^2)	-
26	Grow/finish	-	+ (10^2)	NA
27	Grow/finish	-	-	NA
28	Wean to finish	-	NA	-
Total		7 of 28	15 of 22	3 of 8

[a] The number of HEV genome titer as genome equivalent per 60 ml of manure sample, determined by semi-qualitative RT-PCR.

[b] -, negative for HEV RNA by qualitative RT-PCR; +, positive for HEV RNA by qualitative RT-PCR, thus subjected to semi-quantitative PCR.

[c] Sample not available at the farm.

NA- Not available

Table 2. Swine bioassay results: detection of HEV RNA in feces and anti-HEV serum antibodies in pigs inoculated with manure samples.

Inocula	Route	No. of positive pigs on the following dpi;									Anti-HEV
		0	7	14	21	28	35	42	49	56	56 dpi
Uninoculated	-	0/3[a]	0/3	0/3	NT[b]	NT	NT	NT	NT	0/3	0/3
Lagoon A	Oral	0/3	0/3	0/3	0/3	0/3	0/3	NT	NT	0/3	0/3
Lagoon A	IV	0/3	0/3	0/3	0/3	0/3	0/3	NT	NT	0/3	1/3
Pit B	Oral	0/3	0/3	0/3	0/3	0/3	0/3	0/3	NT	0/3	0/3
Pit B	IV	0/3	0/3	1/3	2/3	2/3	0/3	0/3	NT	0/3	1/3
Positive control	IV	0/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4	0/4	3/4

[a] Number of pigs positive/number of pigs tested
[b] Not tested

Table 3. Pair-wise comparison of nucleotide sequence of the prototype swine HEV (sHEV) and HEV isolates identified from fresh pooled fecal samples collected from 7 pig farms.

	18[a]	12	19	9	21	25	22
sHEV	92[b]	88	90	93	93	93	96
18		91	92	92	94	94	92
12			88	90	90	91	89
19				94	92	91	90
9					94	94	92
21						94	93
25							94
22							

[a] Farm identification number.

[b] Percentage of nucleotide identities in the ORF2 genes.

Table 4. Pair-wise comparison of nucleotide sequence of the prototype swine HEV (U.S. sHEV), the pit B inocula and HEV isolates identified from fecal samples collected at 21 dpi from pigs in the swine bioassay.

	U.S. sHEV	Pig I	Pig II
Pig I	92[a]		
Pig II	91	93	
Pit B Inocula	92	100	93

[a] Percentage of nucleotide identities in the ORF2 genes.

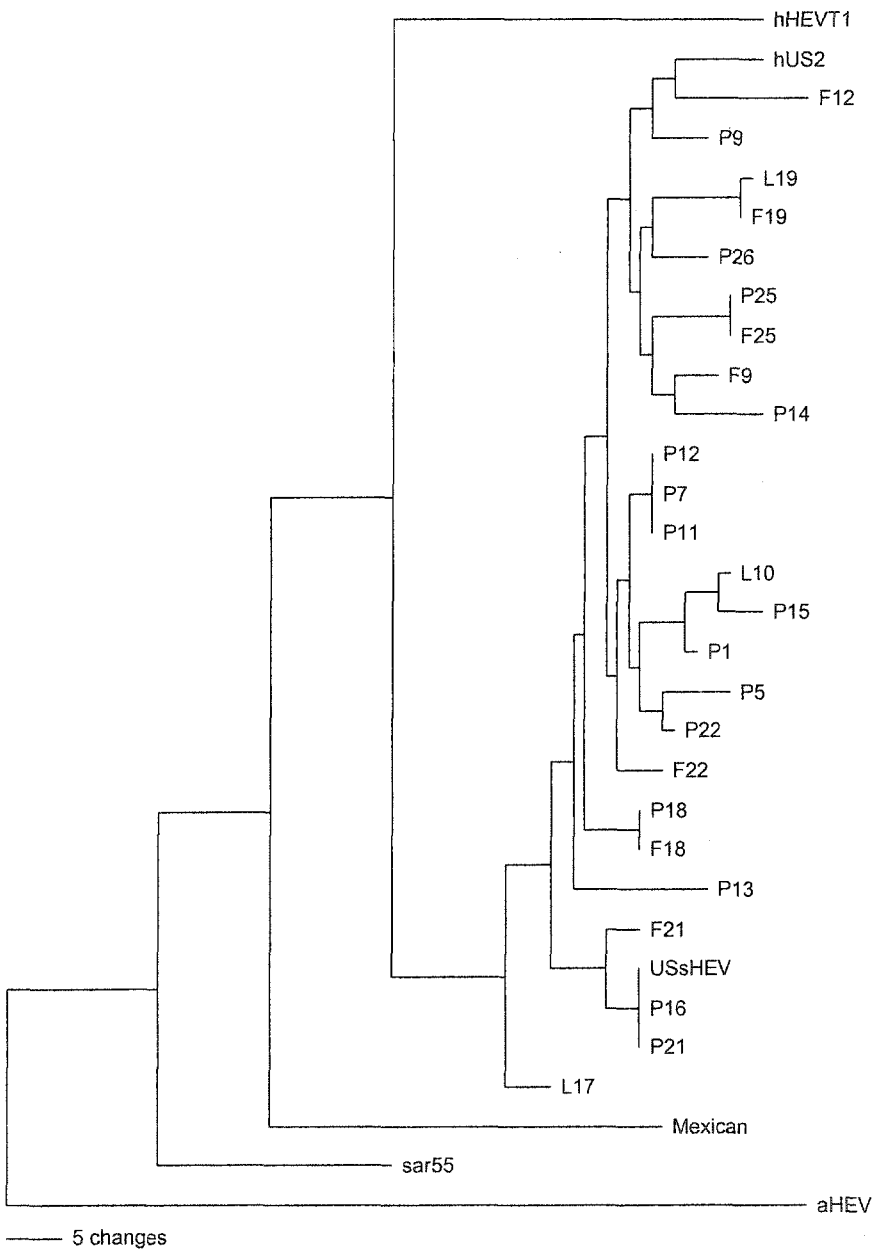

Figure 1. A phylogenetic tree based on analysis of a 304-bp nucleotide sequence of the ORF2 gene of HEV. Representative HEV strains of 4 major genotypes and a recently-classified avian HEV strain were included in the analysis. F, P, and L stands for the HEV sequence amplified from pooled fecal, pit, and lagoon sample, respectively, which are presented in Table 1. The number following the sample initial refers to farm identification in Table 1. A scale bar reflecting the numbers of character state changes is provided for the comparison of genetic distance. The sequence of fecal sample collected from farm # 25 was not available for analysis.

CHAPTER 7.

INFECTION OF PIGS WITH AVIAN HEPATITIS E VIRUS

A paper to be submitted to

Journal of Clinical Microbiology

Chaiyan Kasorndorkbua, Denis K. Guenette, Fang F. Huang,

Pete J. Thomas, Xiang-Jin Meng, S. U. Emerson, R. H. Purcell, and Patrick G. Halbur

Abstract

Cross-species transmission of HEV from chickens to turkeys has been demonstrated; however, attempts to infect rhesus monkeys with avian HEV were unsuccessful. In the present study, we used a pig model to determine if HEV from chickens (avian HEV) or rats (rat HEV) was infectious to pigs. Thirty six, specific-pathogen-free pigs were randomly separated into 4 groups of 9 pigs each. Group 1 was inoculated with HEV-free fecal suspensions and served as the sham-inoculated group. Group 2 was inoculated with rat HEV. Group 3 was inoculated with avian HEV. In the rat and avian HEV groups, 6 pigs were inoculated with the corresponding virus and 3 pigs remained uninoculated and served as contact controls. Group 4 was inoculated with the prototype swine HEV. The inoculation route was intravenous for all groups. Necropsy of 3 pigs from each group was performed on 7, 21, and 35 dpi. In the rat and avian HEV groups, 2 inoculated and 1 contact control pigs were necropsied at each time point. Liver and bile from sham-inoculated pigs were negative

for HEV RNA throughout the study. HEV RNA was not detected in feces from any pigs in the rat HEV group. Pigs inoculated with avian HEV shed HEV RNA in feces from 7 to 28 dpi and were viremic from 7 to 21 dpi. HEV RNA was detected in liver and bile from 1 to 3 pigs (including 1 contact control) in the avian HEV group. One contact control pig in the avian HEV group which was viremic on 7 dpi and shed HEV in feces developed anti-HEV antibodies on 35 dpi. Pigs in the swine HEV group shed HEV in feces from 7 to 28 dpi and 1 pig was viremic on 21 dpi. HEV RNA was detected in liver and bile from 1 pig in the swine HEV group on 21 dpi. Two swine HEV-inoculated pigs seroconverted to HEV on 21 and 35 dpi. Pigs in the sham- and rat HEV-inoculated groups remained seronegative throughout the study. Avian and swine HEV induced subclinical hepatitis; however, the lymphoplasmacytic hepatitis lesions were slightly more severe in the swine HEV group. The findings indicate that avian HEV is transmissible to pigs. This work may open new areas of study in the epidemiology of HEV. Pigs may be an excellent model for comparative molecular and pathogenetic studies of HEV.

Introduction

Hepatitis E virus (HEV) is the primary causative agent of acute, non-A, non-B hepatitis in humans (4). The infection is generally self limiting and typically has no chronic consequences (38). The mortality caused by HEV infection is reportedly low; however in pregnant women the mortality rate may be as high as 25%. Patterns of HEV transmission are different in developing and industrialized countries. In developing countries, epidemics of HEV infection are often associated with fecal contamination of drinking water following heavy rains or flooding (4, 38). In contrast, individuals in industrialized countries usually

contract hepatitis E while traveling to developing countries where HEV infection is endemic (30, 37). In recent years, the emergence of sporadic cases of human HEV infection has been reported in individuals residing in non-endemic countries and with no history of having traveled to endemic countries (3, 22, 27, 32, 41). Subclinical HEV infection may be more prevalent than previously thought particularly in industrialized countries (7, 10).

HEV is a non-enveloped, single-stranded, positive sense RNA virus (38). The virus has currently been designated as a *Hepevirus* in the family *Hepeviridae* (9). HEV has been identified and characterized in pigs and chickens (13, 16, 18, 35, 36). Swine HEV is genetically closely related to human HEV (17, 22, 27, 36, 40, 45, 46). Pigs are thought to be a potentially important source of zoonotic infection of humans with HEV.

Pigs are a natural host of HEV and swine HEV is globally ubiquitous in pig populations (3, 7, 10, 13, 22, 25, 26, 34, 45, 46, 48); however, there is no clinical disease reported in pigs infected with HEV under natural or experimental conditions (15, 36). Natural transmission of HEV in pigs is thought to be via the fecal-oral route and this has been recently confirmed experimentally (29, 48). Cross-species transmission studies have demonstrated that human HEV infects pigs and swine HEV infects non-human primates (15, 35). Individuals who work in close contact with pigs (i.e. pig veterinarians, caretakers, and farmers) are more likely than the general population to have anti-HEV antibodies (8, 33, 34).

Direct evidence of the zoonotic potential of swine HEV has recently been documented in a cluster of cases in which human HEV infection was reported in patients who consumed raw liver from a wild boar (32). HEV found in pig livers sold as food in grocery stores was found to be genetically highly homologous to a virus causing hepatitis E

in humans in Japan (49). Another recent human HEV infection was associated with the consumption of raw meat collected from two wild deer (44).

Avian HEV was first discovered in bile samples of chickens with hepatitis-splenomegaly (HS) syndrome in 2001 (18). Hepatitis-splenomegaly syndrome is a poorly-described disease and the etiology remains unclear (39). The relationship between avian HEV and HS syndrome is not clear since antibodies against avian HEV were also found in healthy chicken flocks (25). Avian HEV is widespread in commercial chicken populations (43). The prevalence of anti-HEV in chickens increases with age and is similar to swine HEV infection in pigs (7, 36, 43). Avian and swine HEV have certain similarities (24). Avian HEV is genetically and antigenically related to swine HEV (16). Analysis of nucleotide sequence revealed approximately 60% homology between the two viruses (25). Avian HEV recovered from chickens also has the ability to cross species barriers and infect turkeys; however, avian HEV was not transmissible to rhesus monkeys (24, 42).

Anti-HEV antibodies have reportedly been found in rodents from different geographical parts of the world (10, 19, 28). Laboratory rats have been shown to be susceptible to experimental infection by a human HEV strain (31). It has been suggested that rats may be an alternative reservoir of HEV (10). Evidence of rat-to-human transmission of HEV was suggested in Nepal where Hepatitis E is endemic (19). The genetic sequence of the rat HEV amplified in that study revealed similarity with genotype 1 HEV strains which predominantly occur in Asia (19). However, HEV in rats in the U.S. remains to be characterized (10).

HEV is a recently discovered virus and the relatively large and diverse species prone to infection with HEV suggest that the virus may readily adapt to new hosts. Chickens, rats,

and pigs are commonly found together on farms across the world. The aim of the present study was to determine if HEV recovered from chickens or rats is infectious to pigs.

Materials and Methods

Virus inocula. The swine HEV inocula used in the present study was prepared from feces collected from pigs intravenously infected with the prototype strain of swine HEV (35). The inocula contained $10^{4.5}$ 50% pig infectious dose (PID_{50}) of HEV per ml, which is equivalent to approximately 10^6 genome equivalent (GE) per ml (35). The avian HEV inocula used in the present study was prepared from bile and feces collected from chickens intravenously inoculated with avian HEV and contained a titer of 5 X 10^5 50% chicken infectious dose per ml (CID_{50}) which is equivalent to approximately 10^6 GE (42). In a pilot study (C. Kasorndorkbua, unpublished data), 2 pigs did not become infected with avian HEV when intravenously inoculated with a HEV titer of 10^4 GE per ml (42) thus a higher titer was used in the current study. The rat HEV inocula originated from the liver of rats from Los Angeles, CA. in the U.S. (10). The rat virus had been serially passage in laboratory rats. The rat liver homogenate was titrated in a bioassay of laboratory rats inoculated with the serial dilution of the original liver homogenate and contained a titer of $10^{5.2}$ 50% rat infectious dose (RID_{50}) per ml. Enzyme linkage immunosorbent assay (ELISA) with the Sar55 HEV strain ORF2 capsid protein as the antigen was used as a marker to confirm HEV infection in the rats. Feces collected from pigs used as negative controls and prepared as 10% fecal suspension (wt/vol) in sterile phosphate-buffered saline (PBS) served as sham inocula for the negative-control. The feces used for sham inocula were confirmed to be free for avian and swine HEV RNA by nested RT-PCR.

Animals and experimental design. Thirty-six, 6-week-old, specific-pathogen-free pigs (*Sus scrofa domesticus*) were used in this study. All pigs were free of swine HEV RNA in feces and seronegative to both anti-avian HEV and anti-swine HEV antibodies prior to inoculation. The negative-control and avian HEV-inoculated pigs were also tested and found to be negative prior to inoculation for avian HEV RNA in feces by avian HEV-specific nested RT-PCR.

The experimental design consisted of 4 groups, including negative-control, rat HEV, avian HEV, and swine HEV-inoculated groups. Each group contained nine randomly selected pigs. Each group was housed in separate room and confined in a pen with raised wire decks. The inoculation route was intravenous for all 4 groups and was performed on a single day. Nine pigs in the negative-control group were inoculated with 1 ml of HEV-free, 10% fecal suspension. The nine pigs in each of the rat and avian HEV group were randomly assigned to 2 sub-groups. In the rat HEV group, six pigs were inoculated with a titer of $10^{4.5}$ RID_{50} of rat HEV inocula and the three remaining pigs served as uninoculated contact controls. In the avian HEV group, six pigs were inoculated with 5×10^5 CID_{50} of the avian HEV inocula and three pigs served as uninoculated contact controls. Nine pigs in the swine HEV group were inoculated with $10^{4.5}$ PID_{50} of the swine HEV.

All pigs were monitored for clinical signs throughout the study. Rectal temperature was recorded every other day through 14 days postinoculation (dpi). Feces and serum samples were collected prior to inoculation and weekly postinoculation until the end of the experiment on 35 dpi. The samples were tested for the presence of HEV RNA by nested RT-PCR and anti-avian HEV and anti-swine HEV IgG antibodies by ELISA. Feces were collected with a sterile Dacron swab and placed in sterile PBS. Fecal swabs and serum

samples were stored at -80° C until tested. Liver and bile samples were also collected from all pigs at necropsy and were tested for HEV RNA by nested RT-PCR. Liver was prepared as a 10% liver homogenate (wt/vol) by a laboratory blender (Stomacher 80, Seward, U.K.).

Examination of gross and microscopic lesions. Necropsy was performed at 3 time points; 7, 21, and 35 dpi. Three pigs from each group were randomly selected for necropsy at each time point. In the rat and avian HEV groups, the three pigs for each necropsy comprised two inoculated and one contact-control pig. Necropsied pigs were grossly examined and a set of tissue samples (liver, gall bladder, mandibular salivary gland, lung, heart, kidney, pancreas, spleen, tonsil, lymph nodes [hepatic, tracheobronchial, and mesenteric], stomach, duodenum, jejunum, ileum, and colon) were collected and fixed in 10% neutral-buffered formalin. Two pieces from each of liver lobes (right lateral, right medial, left lateral, left medial, quadrate, and caudate lobes) were collected. All tissues were then processed, prepared as microslides, stained with hematoxylin and eosin and examined histopathologically. Liver sections were blindly scored as previously described (15). Ten hepatic lobules from each lobe were examined. Hepatitis scores ranged from 0 to 4; where 0 is no inflammation, 1 is 1 to 2 focal lymphoplasmacytic infiltrates per 10 hepatic lobules, 2 is 3 to 5 focal infiltrates, 3 is 6 to 10 focal infiltrates, and 4 is > 10 focal infiltrates.

Detection of swine or avian HEV RNA by nested RT-PCR. RNA extraction was performed by the modified spin column method (QIAamp; QIAgen, Chatsworth, CA) as previously described (1). One hundred-forty µl of samples (fecal suspensions, serum samples, bile, or liver homogenates) were used for RNA extraction. The RNA extract was then immediately used for the reverse transcription reaction and cDNA synthesis by an

incubation of the RNA, a reverse primer, and Superscript II reverse transcriptase (Invitrogen) at 42° C for 1 h.

To detect avian HEV RNA, an avian HEV-specific nested RT-PCR assay was performed as previously described (24). The first-round PCR primers comprised the forward primer FAHEVEp [5'- CATCCACCCCTACAAGCATTGAC-3'] and the reverse primer RAHEVEp [5'-TACGCAACACATCCCCTGACCT-3']. The second-round primers included the forward primer FAHEVEpF [5'-GCCGCTTGGTATGGTTGATTTT-3'] and the reverse primer RAHEVEpR [5'-GGCATCCTCAACCGACATATAC-3']. The second-round PCR reaction produced a PCR product size of 373 base pairs (bp) in length.

To detect swine HEV RNA, a universal nested RT-PCR assay specific for swine HEV was performed as previously described (26). The first-round PCR primers comprised the forward primer F1 [5'-AATTATGCC(T)CAGTAC(T)CGG(A)GTTG-3'] and the reverse primer R1 [5'-CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC-3']. The second-round primers included the forward primer F2 [5'-GTT(A)ATGCTT(C)TGCATA(T)CATGGCT-3'] and the reverse primer R2 [5'-AGCCGACGAAATCAATTCTGTC-3']. The expected second-round amplified PCR product was 348 bp. The amplified PCR products were examined on 1% agarose gel electrophoresis. The PCR products were sequenced to confirm that they were indeed avian HEV sequences.

Detection of anti-HEV immunoglobulin (Ig) G antibodies by ELISA. To detect anti-avian HEV IgG antibodies, a purified truncated avian HEV ORF2 protein expressed in *Escherichia coli* was used as the antigen as previously described (25). A purified 55-kDa truncated recombinant capsid protein from the Sar55 strain of human HEV was used as the antigen to detect the anti-swine or anti-rat HEV IgG antibodies (28, 36). Each serum sample

was tested in duplicate. The final optical density (OD) values were an average of the two ODs. The cutoff value above 0.2, 0.3 and 0.3 was considered as positive for anti-rat HEV, anti-avian HEV and anti-swine HEV IgG antibodies, respectively. Preimmune and hyperimmune swine sera were used as negative and positive controls, respectively.

Statistical analysis. The difference of overall hepatitis lesion scores among inoculated groups and for each different dpi was analyzed by Kruskal-Wallis test using a commercial software (SAS Institute, Cary, NC). Explanatory variables included inoculated groups (nominal variable) and the dpi (ordinal variable). A P value of less than 0.05 was considered significant in all analyses.

Results

Clinical observation. There was no evidence of clinical disease in pigs in any of the inoculated groups (data not shown).

Gross and microscopic findings. No remarkable gross lesions were observed in any of the sham-inoculated or HEV-inoculated groups. Microscopic lesions were confined to the liver and are summarized in Table 1 and Figures 1 and 2. Variably severe, multifocal, lymphoplasmacytic hepatitis was present in 5 of 9 sham-inoculated, 7 of 9 rat HEV-inoculated, 7 of 9 avian HEV-inoculated, and 9 of 9 swine HEV-inoculated pigs. Variably severe, focal hepatocellular necrosis was detected in the lesions of avian and swine-HEV inoculated pigs. There was a significant difference ($P < 0.05$) in the overall mean hepatitis lesion scores. On 7 dpi, the mean hepatitis lesion score of swine HEV-inoculated group was significantly ($P = 0.03$) more severe than those observed on the avian HEV-inoculated group.

The inflammatory foci observed in the livers collected from swine HEV-inoculated pigs were similar in type but larger than those observed in the other groups.

Detection of HEV RNA. Results of the nested RT-PCR assays for HEV RNA in feces, serum, liver, and bile samples tested are summarized in Table 2. None of the liver and bile samples collected from pigs inoculated with sham inocula contained HEV RNA. HEV RNA was not detected in fecal samples obtained from pigs inoculated with rat HEV at 0, 7, 14 or 35 dpi.

All fecal samples from all pigs inoculated with avian HEV (and contact controls) contained HEV RNA beginning on 7 dpi and continuing through 28 dpi. HEV RNA was detected in serum samples from avian HEV-inoculated pigs from 7 to 21 dpi. In the avian HEV group, HEV RNA was detected in serum samples of 5 (including 3 contact controls), 1 (a contact control), and 1 (a contact control) pig on 7, 14, and 21 dpi, respectively. HEV RNA was detected in liver of one pig inoculated with avian HEV necropsied each on 7, 21, and 35 dpi. HEV RNA was detected in bile from 2 avian HEV-inoculated and 1 contact control pigs on 7 dpi, 1 inoculated pig on 21 dpi, and 1 inoculated and 1 contact control pig on 35 dpi.

PCR products amplified from feces collected from a pig inoculated with avian HEV on 7, 14, and 21 dpi, and PCR products amplified from bile samples collected from 3 pigs inoculated with avian HEV on 7 dpi were sequenced. Sequence analysis confirmed that the sequences of the virus recovered from the inoculated pigs aligned with the consensus sequence of the prototype avian HEV used as the avian HEV inocula. The analysis also revealed multiple nucleotide mutations within the sequences of the PCR products.

In the swine HEV group, HEV RNA was found in feces from 4, 6, 5, and 1 pig on 7, 14, 21, and 28 dpi, respectively. HEV RNA was detected in the serum sample of one pig on 21 dpi. Only one pig was positive on 21 dpi for HEV RNA in the liver, while 2 and 1 pigs were positive for HEV RNA in bile samples on 7 dpi and 21 dpi, respectively.

Detection of anti-HEV antibodies. None of the pigs had anti-HEV antibodies prior to inoculation. All pigs in the sham-inoculated group remained seronegative for both anti-swine and anti-avian HEV antibodies throughout the study. All pigs in the rat HEV group also remained seronegative through the duration of the study.

In the avian HEV group, all 6 pigs (4 inoculated and 2 contact controls) remained negative for anti-HEV at 21 dpi. One contact control pig developed anti-HEV by 35 dpi and this pig shed avian HEV in feces from 7 to 28 dpi. The seropositive contact-control pig was viremic on 7 dpi. On 35 dpi, when the anti-avian HEV seropositive pig was necropsied, HEV RNA was detected in the bile; however, liver collected from the pig was negative for HEV RNA.

In the swine HEV group, 2 of 6 pigs developed anti-swine HEV antibodies by 21 dpi and those 2 pigs continued to be seropositive on 35 dpi.

Discussion

The findings of the present study indicate that avian HEV infects and replicates in pigs based on demonstration of HEV RNA in feces, serum, liver, and bile samples of experimentally-inoculated pigs. One of the contact control pigs also developed antibodies against the avian strain of HEV.

Avian HEV recovered from chickens has recently been shown to cross species barrier and infect turkeys (42). Avian and swine HEV are genetically distant (25, 43); however, avian HEV may have ability to adapt to a wide range of host species. The partial sequence amplified from feces collected from one infected pig revealed 4 single nucleotide mutations. The primers used were designed to target the ORF2 sequence which encodes for capsid protein. Thus, the nucleotide mutations may reflect the ability of the virus to readily modify its protein envelop to accomodate different receptors in a variety of host species. These findings indicate that pigs may be used as a model to study the molecular mechanism of virus adaptation to different hosts. Infectious cDNA clones of swine HEV have been demonstrated to be infectious in pigs (23). The use of infectious clone of HEV and a swine model that is susceptible to multiple strains of HEV should facilitate the comparative study of HEV virulence factors and pathogenesis.

Pigs and chickens are commonly raised in close proximity. Both swine and chicken HEV are ubiquitous in their respective host populations (25, 26). It is likely that natural exposure of pigs to avian HEV and likewise chickens to swine HEV occurs frequently in the field. Such conditions would provide opportunities for avian HEV to adapt for replication in pigs and vice versa. It currently remains unknown if swine HEV is transmissible to chickens.

Attempts in our preliminary study to transmit the avian HEV to two pigs using inocula with a titer of 10^4 GE via the intravenous route were unsuccessful (data not shown). Thus, in the present study we used a higher titer of 5 X 10^5 CID_{50} which is equivalent to approximately 10^6 genome equivalent. Inoculation of rhesus monkeys with a similar titer of avian HEV was unsuccessful (24). Dose dependent infection of avian HEV has been demonstrated in experimentally-infected chickens (42).

The inoculation route in the present study was intravenous, although this experimental route is probably unlikely to occur under natural conditions. The intravenous route was used because experimental HEV infection via oral inoculation is known to be less successful (38). It remains to be determined if avian HEV is transmitted via the fecal-oral route as is the case with swine HEV in pigs (29). All avian HEV-inoculated pigs shed the virus in feces from 7 to 28 dpi, and contact control pigs in the avian HEV-inoculated group became avian HEV-infected. Viremia was detected in 3 contact control pigs by 7 dpi, 1 pig by 14, and another on 21 dpi. This suggests that the avian HEV shed by the inoculated pigs was infectious and transmission occurred through direct contact. It is likely, but yet to be confirmed, that transmission occurred via the fecal-oral route since all pigs in the avian HEV-inoculated group shed the virus in feces. However, aerosol or exposure to other body secretions such as saliva can not be ruled out under the experimental conditions of this study.

Avian HEV infection of pigs induced subclinical hepatitis which is similar to what we observed in the present and other studies with swine HEV infection (15, 35). The hepatitis lesions found in the swine HEV-inoculated pigs were similar in type but slightly more severe than those in the avian HEV-inoculated pigs. The mild hepatitis lesions found in the sham-inoculated and rat HEV-inoculated groups are likely non-specific and not related to HEV. This amount of background inflammation in pigs livers is expected (15). There was no evidence that these pigs were infected with the porcine circovirus-2 (PCV-2) or porcine reproductive and respiratory syndrome virus (PRRSV). Porcine circovirus-associated lesions in pigs (granulomatous hepatitis and lymphoid depletion) were not observed in the liver and lymphoid tissues in the pigs in this study and PCV-2 antigen was not detected by

immunohistochemistry (data not shown). All 12 pigs remained seronegative for PRRSV through 35 dpi (data not shown).

The viremic stage of HEV infection is transient (15). It typically follows the onset of virus shedding in feces by 1 week and persists for only 1 to 2 weeks. Only 1 pig in the swine HEV group had detectable HEV RNA in the serum sample and this was at 21 dpi. Viremia was not detected in pigs transfected with a swine HEV infectious clone (23). This may also explain why most pigs in the swine HEV group did not have detectable HEV RNA in serum samples that were collected and tested at weekly intervals.

Hepatitis E virus is thought to replicate in the cytoplasm of hepatocytes (6, 14, 47). The epithelial cells of intrahepatic bile ducts may also support HEV replication (6, 14). Extrahepatic sites of HEV replication also exist and include the intestines, lymph nodes, tonsil, and spleen (6, 47). HEV RNA was found in the liver and bile samples in pigs inoculated with avian HEV. Avian HEV RNA was also detected in the liver and bile samples in a number of pigs. This may reflect the shift and/or different predilection of tissues that avian HEV replicates and might be different from swine HEV.

Serological evidence suggests that rats may serve as a reservoir of HEV in humans (11, 20, 28). In the present study, we attempted to determine if HEV discovered in wild rats was infectious to pigs. The pigs in the rat HEV group did not develop detectable levels of anti-HEV antibodies. The nest RT-PCR used in the current study failed to detect HEV RNA in feces. The primers of the RT-PCR were designed to detect the ORF2 genes of different known strains of human and the prototype U.S. swine HEV in genotype 1, 2, and 3 (26). However, rat HEV remains to be genetically characterized. It is possible that the genetic makeup of HEV in rats is distinctly distant from other U.S. HEV strains and may not be

detectable with the primers used in the present study. HEV is genetically clustered according to geographical regions (22, 26, 27, 40, 45). Rat HEV found in Nepal was genetically related genotype 1 which is a predominant genotype causing epidemics in humans in the country (19). It is speculative that rat HEV found in the U.S. possibly belongs to genotype 3 which includes the US-2 strain of human HEV and the U.S. swine HEV.

In past two decades, emerging viral infections have been reported as zoonotic epidemics in humans and most of the viruses (i.e. sever acute respiratory syndrome [SARS] coronavirus, influenza viruses, or Nipah virus) are RNA viruses (2, 5, 21). These emerging viruses share a common characteristic of an animal reservoir. Current practices of livestock production create favorable conditions for emergence of new viruses and interspecies transmission since the animals are often raised in large and dense populations (21) or are kept in close contact (5). This type of production may increase the propagation of viruses to large numbers, transmission to other hosts in these environments, and prolonged survival and circulation to sustain their existence in environments. Mixed multi-species environments (i.e. live-poultry markets) have been linked to the emergence of cross-species influenza A epidemics between domestic fowl and in-contact humans in Hong Kong (5). In addition, increasing contact between humans and animal reservoirs may facilitate cross-species transmission from susceptible reservoir species to humans (12).

Pigs potentially serve as mixing vessels for zoonotic viral diseases as suggested in influenza A viruses (2). The role of intermediate hosts between a natural reservoir species and humans has been implicated in the recent emergence of SARS coronavirus epidemics (5). Viruses replicating in intermediate host species may adapt to augment a range of susceptible host species. It remains to be determined if avian HEV becomes more virulent or

continues to gain the ability for replication in an extended range of host species following sequential passage in susceptible animals such as pigs.

In summary, we demonstrated that avian HEV was readily transmissible to pigs by intravenous inoculation. Avian HEV from inoculated pigs was infectious and readily transmitted to uninoculated contact-control pigs. Avian HEV induced subclinical hepatitis in pigs and appeared to replicate efficiently in the pigs as HEV RNA was found in feces and bile. The genetic sequence of avian HEV following a passage in pigs revealed a number of single nucleotide mutations and may reflect adaptability to a new host. This may open new avenues of research into the molecular pathogenesis and epidemiology of HEV infection.

Acknowledgments

This work was supported by a grant (to P. G. H.) from the USDA Formula Fund, Pork Checkoff Dollars from the National Pork Board and Iowa Pork Producers Committee, and in part by grants (to X.-J. M.) from the National Institutes of Health (AI01653 and AI46505). The authors thank Dr. Chalermpol Lekcharoensuk for assistance with statistical analysis.

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Table 1. Hepatic lesions in sham, rat, avian, and swine HEV-inoculated pigs

Group	Mean score ± S. D. of hepatitis lesions[a] in pigs (n = 3) on the following dpi			Total mean score ± S. D.
	7	21	35	
Sham-inoculated	0^b	1.00 ± 0.00^b	0.67 ± 0.58^b	0.56 ± 0.53^b
Rat HEV-inoculated	1.00 ± 0.00^b	1.00 ± 0.00^b	0.33 ± 0.58^b	0.78 ± 0.44^b
Avian HEV-inoculated	0.67 ± 0.58^b	1.00 ± 0.00^b	0.67 ± 0.58^b	0.78 ± 0.44^b
Swine HEV-inoculated	2.00 ± 0.00^c	1.00 ± 0.00^b	1.00 ± 0.00^b	1.33 ± 0.50^c

[a] Hepatitis lesions characterized by lymphoplasmacytic inflammation; scores: 0 = no inflammation, 1 = 1 to 2 focal lymphoplasmacytic infiltrates per 10 hepatic lobules, 2 = 3 to 5 focal infiltrates per 10 hepatic lobules, 3 = 6 to 10 focal infiltrates per 10 hepatic lobules, 4 = > 10 focal infiltrates per 10 hepatic lobules.

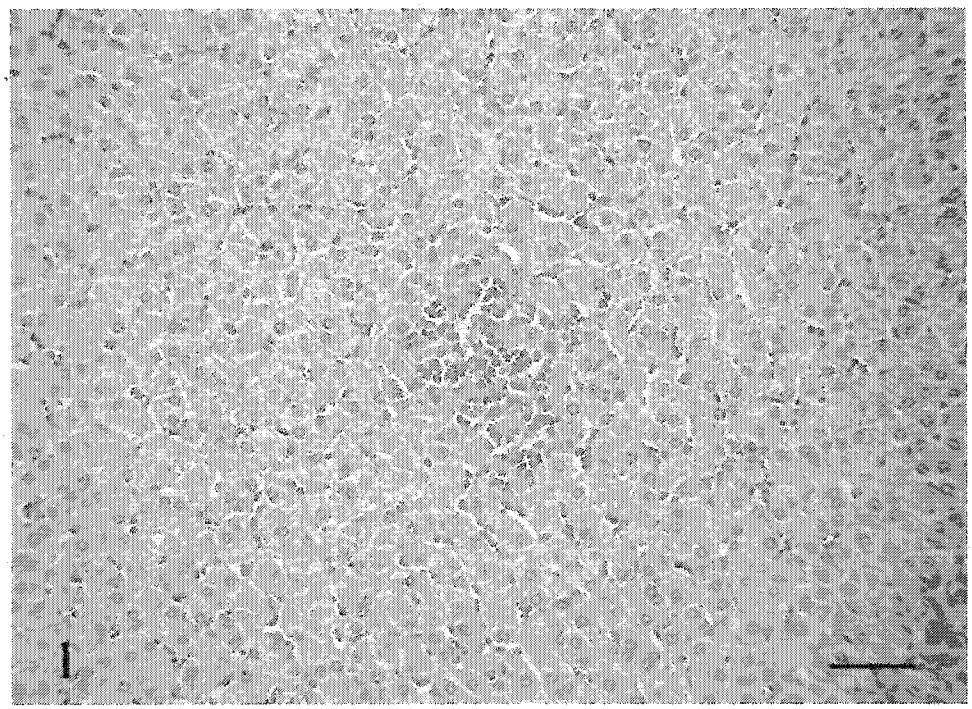
[b,c] Values with different supercripts (*b* or *c*) are significantly different ($P < 0.05$) from other values in the same column.

Table 2. HEV RNA in feces, serum, liver, and bile of sham- and HEV-inoculated pigs

Inocula	Sample	No. of positive inoculated pigs (no. of positive contact control pig) per no. tested on the following dpi;					
		0	7[a]	14	21[a]	28	35[a]
Sham							
	Feces	0/9	0/9	NT[b]	NT	NT	0/3
	Serum	NT	NT	NT	NT	NT	NT
	Liver	NT	0/3	NT	0/3	NT	0/3
	Bile	NT	0/3	NT	0/3	NT	0/3
Rat HEV							
	Feces	0/9	0/9	0/6	NT	NT	0/3
	Serum	NT	NT	NT	NT	NT	NT
	Liver	NT	NT	NT	NT	NT	NT
	Bile	NT	NT	NT	NT	NT	NT
Avian HEV							
	Feces	0/9	6(3)/9	4(2)/6	4(2)/6	2(1)/3	0/3
	Serum	NT	5(3)/9	1(1)/6	1(1)/6	0/3	0/3
	Liver	NT	1(0)/3	NT	1(0)/3	NT	1(0)/3
	Bile	NT	2(1)/3	NT	1(0)/3	NT	1(1)/3
Swine HEV							
	Feces	0/9	4/9	6/6	5/6	1/3	0/3
	Serum	NT	0/9	0/6	1/6	0/3	0/3
	Liver	NT	0/3	NT	1/3	NT	0/3
	Bile	NT	2/3	NT	1/3	NT	0/3

[a] dpi on which necropsy was performed.

[b]Not tested, samples not collected or tested on this dpi.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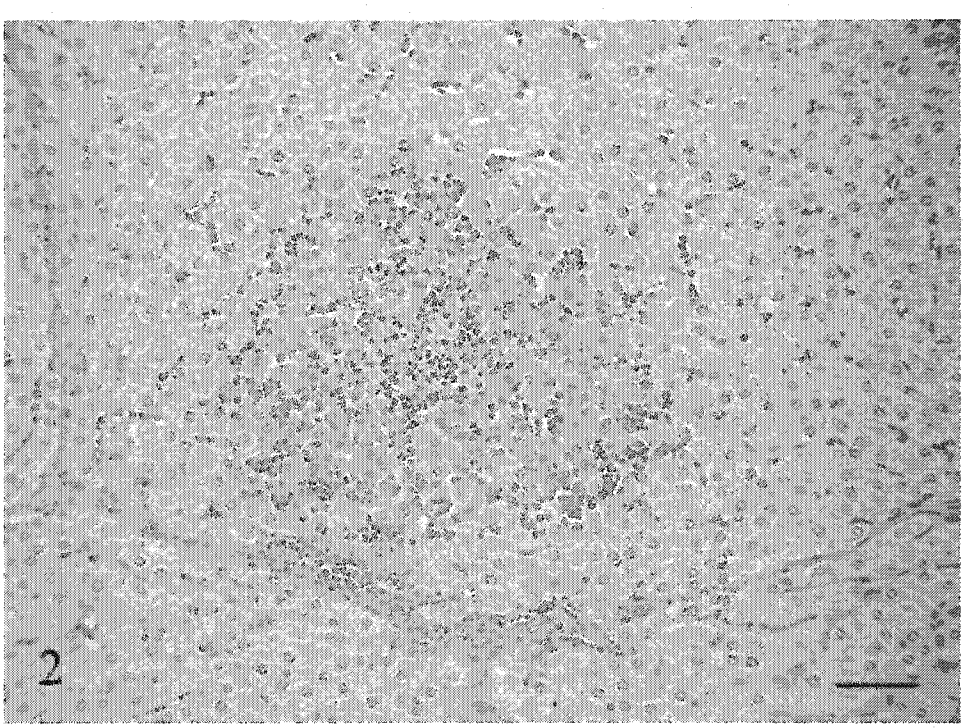
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CHAPTER 8.

GENERAL CONCLUSIONS

Swine HEV causes a subclinical hepatitis in growing pigs (27). HEV-infected pigs became viremic for a relatively short period of 1 to 2 weeks. The viremia occurs 1 to 3 weeks postinfection and fecal shedding typically precedes the viremia by 1 week and persists for 3 to 6 weeks. The presence of HEV in feces serves as a major source of the virus for transmission to contact animals and people and contamination of the environment. Following the viremic state, anti-HEV IgG antibodies first appear by 5 weeks postinfection and persist for several months. There were no remarkable macroscopic lesions in swine HEV-infected pigs. HEV-associated microscopic lesions in growing pigs are characterized by mild multifocal lymphoplasmacytic hepatitis with focal hepatocellular necrosis. The clinical, virological and histopathological pattern of HEV infection in pregnant adult pigs was similar to that reported in growing pigs. *In utero* transmission did not occur in HEV-infected pregnant pigs and HEV infection of the dam did not induce evidence of reproductive failure or disease or lesions in offspring born to the HEV-infected pregnant pigs. These findings suggest that HEV is probably not responsible for reproductive failure in pigs. Piglets that suckled HEV-infected dams acquired anti-HEV IgG antibodies through colostrum intake and the passive antibodies waned to undetectable levels by 2 months of age. Hepatitis E virus-free pigs were derived by early weaning of the piglets (18 days of age) from the dams. This is important information for producers or researchers interested in establishing HEV-free pig sources.

The fecal-oral route appears to be the primary mode of HEV transmission in pigs. HEV is readily transmitted through direct contact, although effective transmission apparently

requires a high number of infective virus particles and/or repeated exposure via the fecal-oral route. Experimental HEV inoculation through the oral route with a single dose of 10^3 or 10^6 GE in a previous study failed to sufficiently induce HEV infection while repeated oral inoculation for three consecutive days was sufficient in inducing HEV infection in our subsequent study. Transmission via the aerosol route, contaminated needles, or exposure to body secretions such as saliva appeared to be unlikely based on the findings of the third experiment.

Liver from pigs in the early stages of HEV infection was found to be a potential xenozoonotic threat to transmit HEV. Pigs inoculated intravenously with liver homogenates collected from other pigs in the early stages of infection developed HEV infection as determined by the presence of HEV viremia and development of anti-HEV antibodies. The risk of xenozoonoic HEV infection by using heart or pancreatic tissues from HEV-infected pigs is minimal, if it exists at all, since HEV was not detected by the RT-PCR in these tissues and the swine bioassay further confirmed that homogenates of heart or pancreas contain no detectable or infectious HEV. The RT-PCR used in the first study appears to be a suitable and sensitive diagnostic tool to detect HEV-infected livers and thus avoid the risk of xenozoonotic transmission via xenotransplantation.

Growing evidence of HEV as a food-borne zoonotic infection has been recently documented (54, 83, 100). Consumption of raw liver collected from a wild boar caused a cluster of HEV infections in Japanese human patients (54). This is in accordance with the findings of our swine bioassay where liver collected from HEV-infected pigs contained viable virus and was infectious to pigs. Another recent report of an incidence of human HEV infection indicates that the consumption of raw meat collected from wild deer may also be

responsible for HEV infection (83). In contrast, pork loin collected from pigs during active HEV infection was found to be free of detectable HEV. It is possible that the HEV present in the deer meat may be due to fecal contamination from the deer or people who handled the meat. The pattern of HEV infection in wild deer remains unknown and awaits further investigation and characterization.

HEV is heterogeneic and geographical clusters of heterogeneous genotypes have been globally reported (20, 40, 71). The results of our fourth study further confirm such conclusions. HEV was found in fresh pig feces and from stored pig manure collected from concrete pits or earthen lagoons on pig farms in Iowa. The partial genetic sequence analysis of HEV amplified by RT-PCR in the study indicated that all HEV isolates in the study clustered within the genotype 3 of HEV which is mainly located in North America and contains the prototype U.S. swine and human US-2 strain of HEV. These isolates were genetically distant from other genotypes of HEV found on other continents of the world. HEV isolated from pig feces and from manure in storage facilities on the same farms tended to be more closely related to each other than to HEV isolates from different farms. This suggests that feces and manure storage facilities (pits and lagoons) may be an important source of contamination of naïve pigs, other domestic animal species, and the environment with HEV.

The fifth study demonstrated that avian HEV infected and replicated in pigs. HEV RNA was detected in the liver, bile and feces of pigs inoculated with avian HEV. One of 3 inoculated pigs developed IgG antibodies specific to avian HEV ORF2 antigen by 35 dpi. The pattern of virus shedding in feces and viremia of avian HEV infection in pigs is similar to that induced by swine HEV. Like swine HEV, the avian HEV induced a subclinical

hepatitis in pigs and the severity of the microscopic lesions was less severe than observed with swine HEV. It is interesting that the nucleotide sequence of the amplicon found in feces and bile collected from a pig infected with the avian HEV revealed a few single point mutations when compared to the sequence of the original virus in the inocula. The findings may reflect the adaptability of avian HEV to replicate in pigs. Although avian HEV may be able to cross species barriers to infect pigs, the avian HEV appears to be less likely a potential zoonotic infection than the swine HEV since the avian HEV failed to experimentally infect rhesus monkeys whereas swine HEV was able to induce infection and lesions in nonhuman primates (38, 59). It appears that rat HEV was not capable of infecting pigs. None of three rat HEV-inoculated pigs developed anti-HEV IgG antibodies specific to genotype 3 HEV strains.

In summary, HEV causes a subclinical hepatitis in both growing pigs and pregnant gilts. Liver and feces from HEV-infected pigs are the primary source of HEV transmission. Natural transmission of HEV in pigs is primarily via the fecal-oral route. Liver transplantation also poses a risk of HEV transmission in humans. Consumption of uncooked pig liver may result in clinical HEV-induced disease in humans. HEV is widespread on pig farms and pig manure may be a source of contamination to the environment and for HEV transmission. Cross-species infection of pigs with avian HEV has now been demonstrated and may open new avenues of research into the molecular pathogenesis and epidemiology of HEV.

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ACKNOWLEDGMENTS

I am most grateful to my major professor, Dr. Patrick Halbur who has provided academic advice and financial support throughout my graduate study. I am very grateful to my Ph.D. committee members: Drs. Mark Ackermann, Steve Sorden, Eileen Thacker, and James Roth for their valuable advice towards my graduation. I would also like to thank Dr. Michael Wannemuehler for attending my final oral examination as a substitute committee member.

I would like to express my gratitude to Dr. Xiang-Jin Meng and his research team, Fang Huang and Denis Guenette for helpful advice and extensive technical support.

My appreciation also goes to Dr. Ryan Royer, Dr. Alex Nunez, Dr. Tanja Opriessnig, Pete Thomas, and Josh Bowden who have assisted with the animal experiments.

I would like to thank the Faculty and Staff of Veterinary Diagnostic Laboratory, Iowa State University for the pleasant and supportive working environment throughout the years.

Dr. Porntippa and Dr. Chalermpol Lekcharoensuk have offered me assistance and advice whenever I asked them for help. To them, I am very thankful.

I am in debt to all of my teachers who taught me with the knowledge that have helped me through this extensive studying time of my life.