Mechanism of Pathogenesis and Replication of an Avian Strain of the Hepatitis E Virus in a Chicken Model

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

In
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April 16, 2007
Blacksburg, VA

Keywords and abbreviations: hepatitis E, hepatitis E virus, HEV, avian HEV, pathogenesis, replication, extrahepatic replication, avirulent avian HEV
Mechanism of Pathogenesis and Replication of an Avian Strain of the Hepatitis E Virus in a Chicken Model

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Abstract

Hepatitis E is an acute, enterically transmitted disease of public health importance in both developing and industrialized countries of the world. Hepatitis E virus (HEV), the causative agent of hepatitis E, is a positive-sense, single-strand, non-enveloped RNA virus. The mechanism of pathogenesis of HEV is poorly understood due to the lack of an in vitro cell culture system and an ideal animal model system. Novel strains of HEV, swine HEV and avian HEV, were discovered and were found to be antigenically and genetically related to human HEV. Non-human primates and swine models have been used to study HEV pathogenesis with little success. With the discovery of avian HEV and its association with a hepatic disease (Hepatitis-Splenomegaly syndrome), chickens provide an excellent small homologous animal model system to study this important virus. Recent identification of an apparently avirulent strain of avian HEV from healthy chickens warrants studies to understand the genetic basis of attenuation. The objectives of this dissertation were to utilize chickens as a model system to study the pathogenesis and replication of avian HEV under the natural route of infection, to identify potential extrahepatic replication sites, to determine and analyze the complete genomic sequence of the avirulent strain of avian HEV, and to study the comparative pathogenesis of the two isolates of avian HEV, the prototype pathogenic and avirulent strains of avian HEV.

We attempted to experimentally infect specific-pathogen-free (SPF) adult chickens by the natural fecal-oral route in order to systematically study HEV
pathogenesis and replication and to characterize the clinical course and pathological lesions associated with avian HEV infection. Sixty-week-old, specific-pathogen-free (SPF) chickens were each inoculated with 5 x 10^{4.5} 50% chicken infectious dose of avian HEV by oronasal route and IV route. All oronasally- and IV- inoculated chickens seroconverted to avian HEV antibodies by 21 days post-inoculation (DPI) and fecal virus shedding was detected variably from 1 to 20 DPI in the IV group, and from 10 to 56 DPI in the oronasal group. Avian HEV RNA was detected in serum, bile, and liver samples earlier during the course of infection in IV-inoculated chickens than in oronasally-inoculated ones. Gross liver lesions including subcapsular hemorrhages and enlargement of right intermediate lobe and microscopic hepatic lesions in the liver characterized by lymphocytic periphebitis and phlebitis were observed in inoculated chickens. Slight elevations of plasma liver enzyme lactate dehydrogenase were also observed in infected chickens. This is the first report of experimental HEV infection via its natural route in a homologous animal model system.

Very little is known about HEV pathogenesis and it has been hypothesized that HEV replicates in tissues other than liver. The replicating negative-strand viral RNA was detected by negative-strand-specific RT-PCR in liver, serum, colorectum, cecum, jejunum, ileum, duodenum and cecal tonsils, but not in other non-GIT tissues. Immunohistochemistry using an avian HEV capsid protein-specific anti-peptide antibody revealed positive signal in liver and GIT tissues including colorectum, jejunum, ileum, cecum, cecal tonsils and pancreas. The detection of avian HEV capsid antigen and replicative negative-strand viral RNA in the GIT tissues indicates that HEV replicates in the GI tract following infection by fecal-oral route. This is the first report of extrahepatic
sites of HEV replication in experimental HEV infections via natural route of inoculation in a homologous animal model system.

The complete genomic sequence of an avirulent strain of avian HEV was determined using primer walking strategy. The full-length genome of the avirulent strain is 6649 nucleotides (nts) in length and has a nucleotide sequence identity of 90.1% with the prototype pathogenic strain. Numerous non-silent mutations were observed in ORF1, the region coding for the nonstructural proteins including the methyltransferase, protease, helicase and RNA-dependent RNA polymerase (RdRp). Six unique non-silent mutations were identified in the capsid-encoding ORF2 region and the ORF3 had four non-silent mutations. Phylogenetic analysis based on full-length genomic sequence revealed that the avirulent strain is clustered together with the pathogenic avian HEV and represents a branch distinct from mammalian HEVs. The identification of 6 unique non-silent mutations in the capsid gene, together with the mutations in other coding regions of the avirulent avian HEV suggests that they may play a potential role in attenuation.

In order to study the comparative pathogenesis between the pathogenic and avirulent strains of avian HEV, an infectious stock of the avirulent avian HEV was generated and infectivity titer was determined to be $5 \times 10^{2.5}$ CID$_{50}$ per ml by experimentally infecting young SPF chickens. To study comparative pathogenesis, six-week-old SPF chickens were inoculated with one of two strains of avian hepatitis E viruses, pathogenic avian HEV recovered from a chicken with HS syndrome and avirulent avian HEV isolated from a healthy chicken. Most of the chickens seroconverted by 3 weeks post-inoculation (wpi) in both pathogenic avian HEV and avirulent avian HEV groups. Avian HEV RNA was detected in feces and serum of the chickens from
both of the inoculated groups from 1 wpi. Microscopic liver lesions included lymphocytic periphlebitis and phlebitis the overall hepatic lesion mean score was higher for the pathogenic avian HEV group compared to the avirulent avian HEV and control groups, suggestive of attenuation. Additional studies with higher infectious dose are warranted to fully understand the differences in the pathogenicity associated with the two strains of avian HEV.

In summary, we experimentally infected SPF chickens with avian HEV by natural route to study the systematic pathogenesis and replication. We identified non-liver replication sites of avian HEV in a chicken model. We determined the complete genomic sequence of an apparently avirulent strain of avian hepatitis E virus and identified major genetic differences compared to the prototype pathogenic strain of avian HEV. We also studied the comparative pathogenesis of avian hepatitis E virus isolates from a chicken with HS syndrome and from a healthy chicken by experimental infections in young SPF chickens. The results from this dissertation research have important implications for the understanding of HEV pathogenesis.
Dedication

To my husband Jaganmohan R. Kamineny,

son Pranay R. Kamineny and daughter Neha R. Kamineny.
Acknowledgements

Firstly, I would like to express my sincere gratitude to my advisor Dr. X.J. Meng for his guidance, support, encouragement and inspiration. My graduate life under his mentorship has been very rewarding and fulfilling, and I cannot imagine having a better advisor. He will always be my role model as a scientist and mentor.

I would like to thank Dr. Toth for his friendly advice and support at all times. I am thankful to Dr. Pierson for help with the chicken experiments, and also for insightful discussions. My thanks are due to Dr. Bob Duncan for help with histopathology, and also for constructive advice. I thank Dr. Jake Tu for expert suggestions and stimulating new ideas. Thanks are also due to Dr. LeRoith for evaluation of histopathology. I am immensely grateful to Dr. Wen Li for help with immunohistochemistry and friendly advice. I sincerely thank Dr. Sriranganathan for always cheering me up when I needed it the most.

I will always cherish the company of my lab colleagues and staff for making my stay in the lab a memorable one. I appreciate the help of Dr. Fang-Fang Huang, Dr. Zhifeng Sun, Denis Guenette, Nicole Juhan, Dr. Kijona Key, Dr. Yao-Wei Huang, Dr. Martin Fenaux, Sumanth, Jennifer DiCristina and Alicia Feagins. Many thanks to Sheela Ramamoorthy for being a good friend and helping me in many ways. I am thankful to all CMMID people especially Zhenquan Jia, Kay Carlson, Nancy Tenpenny and Alba Hall. I would like to recognize Oscar Peralta’s technical help with immunohistochemistry.

I acknowledge the services of animal care personnel and glassware personnel, Steve Salmon, Pamela Mohr, Debbie and Doris. Thanks to Kathy Lowe and Mary
Mainous for technical help. I would also like to recognize Dr. John Lee and Dr. Roger Avery for the financial support.

I am very fortunate to be blessed with a wonderful family. I cannot put in words the appreciation for the unwavering support, sacrifice, wisdom, and devotion of my husband. He is the best husband in the world and I am always grateful to him for inspiring me to get into graduate school, for believing in me and for always being there for me. I am also grateful to my kids, Pranay and Neha for being the driving force of my life. I am also fortunate to have a brother-in-law, K.V. Ramesh Reddy, who always supported and encouraged me in pursuing my research career and took pride in my achievements. I am also thankful to my parents and siblings, Madhavi, Ravi and Ramu for their support. I appreciate my parents-in-law for their encouragement in all ways.

This study was supported by grants from the National Institutes of Health (AI 01653, AI 46505, and AI 50611 and AI065546) and from the U. S. Department of Agriculture National Research Initiative Competitive Grant Program (NRI 35204-12531). We acknowledge the copyright permission to use the article (Chapter 4) from Journal of General Virology.
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General Introduction

Hepatitis E virus (HEV), the causative agent of hepatitis E, is responsible for the majority of enterically transmitted cases of non-A and non-B hepatitis (9, 113, 114). Hepatitis E is epidemic and endemic in many developing countries. Sporadic cases of acute hepatitis E have also been reported in industrialized countries including the United States (91, 93, 94, 113, 119, 120). The disease mostly occurs in young adults. Although the mortality rate is generally low, it can reach up to 15-25% in infected pregnant women (1, 68, 76). The primary mode of HEV transmission is thought to be the fecal-oral route and waterborne epidemics are the most explosive form in developing countries of Asia and Africa (110, 114).

Antibodies to HEV have been detected in a significant proportion of individuals in industrialized countries like US though the incidence of HEV is only sporadic in these countries (95, 99, 119, 120). The discoveries of first animal strains of HEV, swine HEV from pigs and avian HEV from chickens, suggest the existence of animal reservoirs of HEV and leads to the conclusion that hepatitis E is a zoonotic disease (41, 91, 93).

Avian HEV was identified and characterized from chickens with hepatitis-splenomegaly (HS) syndrome in United States (52). Like swine HEV, avian HEV is also genetically and antigenically related to human HEV. Unlike swine HEV, however, avian HEV is associated with a hepatic disease (HS syndrome) (52, 55). Extra hepatic sites of replication of HEV were reported in a swine model under experimental IV inoculation (14). The main constraints for studying HEV are the lack of an in vitro cell culture system and a practical animal model. The discovery of avian HEV and its association
with a hepatic disease provides a homologous small animal model system to study HEV pathogenesis and replication. The chicken model also affords an opportunity to identify extrahepatic sites of HEV replication. Recently, another isolate of avian HEV, the avirulent avian HEV, was isolated from an apparently healthy field chicken. The avirulent strain does not cause a clinical disease under field conditions and therefore, it is important to determine the complete sequence of the avirulent avian HEV and investigate any differences in pathogenicity with respect to pathogenic avian by performing a comparative pathogenesis study.
Chapter 1
Literature Review

Hepatitis E disease

Hepatitis E is an acute, enterically transmitted disease characterized by jaundice, anorexia, malaise, fever, nausea and abdominal tenderness (38, 59, 75). Hepatitis E is epidemic and endemic in many developing countries due to poor sanitation and contaminated water supply. Sporadic cases of acute hepatitis E have also been reported in industrialized countries including the United States (112, 119, 120). It mainly affects young adults and pregnant women, causing a 15-25% mortality in infected pregnant women (76). The mortality rate in general population is about 1% (38).

History of Hepatitis E

Major epidemics of enterically transmitted non-A, non-B hepatitis cases of Hepatitis E occurred in the developing countries of Asia, Africa and North America (15, 38, 112). A non-A, non-B hepatitis etiological agent was first recognized in 1980 in a retrospective study involving the testing of sera from infected patients during water-borne epidemics from mid 50’s to early 90’s (9). However, the hepatitis E virus (HEV) was first identified in the feces of an experimentally infected volunteer by immuno-electron microscopy in 1983 (15). HEV was initially proposed to belong to Caliciviridae family on the basis of electron microscopy (21, 22). The fecal material from a Burmese patient was passaged in cynomolgus macaques, cloned and sequenced (115, 130), followed by sequencing of a diverse strain from Mexico (54). Experimental HEV transmission to primates (12, 15, 23) and domestic pigs (16) was reported and primate models were the
preferred animal models to study HEV. Meng et al., discovered the first animal strain of HEV, the swine HEV, from commercial swine in USA and the reported sequence was significantly different from HEV isolates from Asia and Mexico (98). Since then, many strains of HEV were isolated from pigs from different geographical regions of the world along with human strains (24, 26, 27, 29, 31, 32, 45, 53, 54, 60, 81, 89, 94, 100-102, 106, 122, 124, 129, 146, 152, 153). Sporadic cases of acute hepatitis E have also been reported in industrialized countries including the United States (112, 119, 120).

The Virus

Hepatitis E virus is a spherical, non-enveloped, positive-sense, single-stranded RNA virus with a size of 27-34 nm in diameter with a genome size of about 7.2 kb (38). The genome contains three partially overlapping open reading frames (ORFs) and short 5′ and 3′ nontranslated regions. ORF1, the largest of the three, encodes nonstructural proteins, the putative functional domains like methyl transferase, papain-like cysteine protease, helicase and RNA-dependent-RNA polymerase (RdRp). ORF2 encodes the putative capsid protein and ORF3, which partially overlaps ORF2, encodes a small cytoskeleton-associated phosphoprotein (38, 112, 114). HEV doesn’t grow well in cell culture (38) and lack of an established in vitro cell culture system has been a major impediment in the study of HEV.

Classification

Based on common morphological and physiochemical characteristics, HEV was originally classified in the family *Caliciviridae* (108). However, based on sequence
comparisons and phylogenetic analyses, it appeared to be more closely related to Togaviridae (20, 73). Eventually it was declassified from Caliciviridae and placed in a new genus, Hepevirus, belonging to the family Hepeviridae (35).

Genotypes

Based on sequence and phylogenetic analyses, HEV is divided into four major genotypes (38, 98, 130, 145). HEV strains from Asia and Africa belong to genotype 1, a single strain of HEV from Mexico constitutes the genotype 2. Human and swine HEV strains from U.S. Canada, Europe and Japan are in genotype 3, and human and swine HEV isolates from China, Japan and Taiwan form genotype 4 (38, 82). Genotype 1 and 2 HEV strains are responsible for epidemics in Asia and Africa, while genotypes 3 and 4 are apparently attenuated strains and responsible for sporadic cases (38). A novel HEV strain, the avian HEV, was isolated from chickens with Hepatitis-Splenomegaly (HS) syndrome in the United States. Sequence comparison and phylogenetic analyses of avian HEV revealed that it is the most divergent of the HEV strains with about 50% sequence identity (56). Recently, another strain of avian HEV, avirulent avian HEV, was isolated from apparently healthy chickens (125). It is hypothesized that the two avian HEV strains belong to the putative genotype 5.

Molecular characteristics

Due to the lack of a cell culture system, most of the knowledge of molecular aspects of HEV was obtained by in vitro expression of recombinant proteins (36). ORF1 located at 5' end encodes non-structural proteins involved in RNA synthesis like guanylyl
transferase, methyl transferase (83) and RNA-dependent RNA-polymerase (3, 47). The enzyme activities of guanylyl transferase and methyl transferase are required for the synthesis of cap structure. The 7mG cap structure found in a majority of eukaryotic mRNAs was also identified in HEV (62, 157). Though cap structure was demonstrated to be required for infectivity of HEV recombinants in vivo (40) and replication of HEV replicon expressing green fluorescent protein in vitro (36), capping was reported to be not essential for HEV RNA replication in HepG2 cells in vitro (104). The cap structure is thought to facilitate translation but is not a necessity. The highly conserved GDD motif in the active site of RdRp was reported to be important for replication and a 3' cis-reactive element overlapping 3' end of capsid gene and a part of 3' NCR for viral viability (3, 36, 40). The full-length protein encoded by ORF1 is reported to accumulate as an unprocessed 186 kDa protein, two processed 78 and 107 kDa products or processed products of 35 and 40 kDa (6, 38, 118).

ORF2 gene encodes capsid protein of about 660 amino acids. The capsid protein has a signal sequence and three potential glycosylation sites, the biological functions of which are not known (38). Recombinant ORF2 protein when expressed in insect cells are truncated and form virus-like particles (VLPs) (79). It is hypothesized that the N-terminal region of the capsid protein interacts with genomic RNA (57). Several antigenic sites including a neutralization epitope from 452 to 617 residues, are present in the C-terminal region (90).

The ORF3 encodes a small 123 amino acid cytoskeleton-associated phosphoprotein (154). ORF3 is not required for replication, virion assembly or infection in vitro (37), but is required for in vivo infectivity (58). The third in-frame AUG in the
ORF2-ORF3 junction region is the initiation site for ORF3 translation and is required for virus infection \textit{in vivo} (58). The phosphorylated form of ORF3 protein was reported to interact with the non-glycosylated form of ORF2 capsid protein (142). It binds SH3 domain-containing proteins and activates mitogen-activated protein kinase in cell signaling pathway, suggesting that ORF3 protein has a regulatory function (74). ORF3 protein was also reported to expedite the export of $\alpha_1$-microglobulin from hepatocytes (143). ORF3 is thought to be a viral regulatory protein rather than a structural protein (37), and its role in \textit{in vivo} infectivity indicates that it is important for viral viability and pathogenesis.

\textbf{Seroepidemiology and transmission}

Hepatitis E is epidemic and endemic in many developing countries and sporadic cases of acute hepatitis E have been reported in industrialized countries including United states, Japan and European countries (38, 88, 92-95, 98, 99, 119, 120, 151, 155). Much of the early knowledge of seroepidemiology of HEV was obtained from serosurveys that were conducted in countries like India where the HEV epidemics were frequent. Seroprevalence of anti-HEV antibodies was less frequent in young children compared to adults in India (13). In contrast to this report, anti-HEV antibodies were common in young children and adults in Egypt, where only sporadic cases of Hepatitis E occur (43). Relatively high seroprevalence rates were reported in industrialized countries like Japan (4-7 \%) and United States (15-20 \% in blood donors) (99, 134, 148). The urban sewage samples from non-endemic areas like France, Spain, Greece, Sweden and United States tested positive for HEV (34), suggesting that the healthy populations of the non-endemic
areas may be exposed to other animal strains of HEV that do not manifest any clinical symptoms.

The primary mode of HEV transmission is thought to be fecal-oral route (9, 15). Most explosive forms of HEV epidemics in the developing countries of Asia and Africa are waterborne and are caused by contamination of drinking water with fecal material (9, 112, 114). Unlike other enterically transmitted viral hepatitis like hepatitis A, person-to-person transmission is not common (2, 67). Vertical transmission of HEV is rare, and the infants born to the HEV positive mother were reported to be infected with significant perinatal morbidity and mortality (70, 77). Food-borne HEV infections due to the consumption of undercooked shell fish and meat products have been reported (1, 87). Undercooked pig liver and deer meat were the source of acute hepatitis E infection in Japan (132, 133, 151). Commercial pig livers in the grocery stores in United States were contaminated with HEV and the recovered virus was found to be infectious (42). Post-transfusion hepatitis has also been documented (71, 86). Travel to endemic regions was implicated in the sporadic cases of Hepatitis E in non-endemic regions (72) and contact with infected animal was also a risk factor for HEV infection (78).

Pathogenesis

Due to lack of an efficient cell culture system and a practical animal model, very little is known about the pathogenesis of HEV. Most of the scant knowledge was obtained through studies in non-human primates.

The virus first appears in the liver, followed by viremia. Very high concentration of the virus is found in bile, and is shed in the feces (38). It is unclear how the virus
reaches the liver, as HEV is transmitted by fecal-oral route. It has been suggested that the liver damage is caused by the immune response to the invading virus, and not directly by the replication of the virus in the liver (112). It is believed that the virus first replicates in the gastrointestinal tract (GIT) following oral ingestion and then reaches the liver. Extrahepatic sites of replication were demonstrated in a pig model (147). Replicative, negative-strand HEV RNA was detected in small intestine, lymph node and colorectum. Similarly, extrahepatic sites of replication were also reported in pigs that were naturally infected by swine HEV (30).

Viremia and fecal shedding of virus are followed by humoral immune response, which coincides with appearance of microscopic lesions in the liver (38, 112) suggesting that hepatic damage is immune-mediated (123). Viremia is transient and fecal shedding occurs for 3-4 weeks (28, 33, 80, 136). IgM anti-HEV antibodies appear first, followed by IgG anti-HEV antibodies. IgM anti-HEV titer declines in 2-3 months (28) but IgG anti-HEV can last up to 14 years (25, 33, 69, 80, 85). However, in a study on acute hepatitis E in Egyptian children, anti-HEV IgG was reported to disappear in 6-12 months after infection (46). Serum levels of liver enzymes like alanine aminotransferase (ALT) were elevated in the infected animals (38). Microscopic lesions consistent with acute viral hepatitis were observed in liver biopsies of 2 rhesus monkeys inoculated with swine HEV at the same time point as the liver enzyme elevations (97). Microscopic lesions in liver include focal necrosis and inflammation (112). Biopsy specimens from HEV patients show non-specific inflammation or canalicular bile stasis with pseudoglandular arrangement of hepatocytes around bile canaliculi, which is called cholestatic form and is characteristic of HEV (149).
The incidence and severity of hepatitis E (HEV) is high in pregnant women with 15-25% mortality (76, 109) with the manifestation of fulminant hepatitis in 3rd trimester (76), which was not reproducible in pregnant rhesus monkeys (7, 140). It is hypothesized that HEV might precipitate pregnancy-associated eclampsia with disseminated intravascular coagulation in liver and kidneys, leading to death due to hepatic and renal failure (59). The enhanced sensitivity of pregnant women to endotoxin-mediated damage (called Schwartzman-like phenomenon) is also speculated to be responsible for the high mortality of pregnant women (45, 59, 109).

**Clinical symptoms**

The incubation period of hepatitis E is 2-8 weeks (66). Majority of HEV infections may be asymptomatic or may exhibit flu-like anicteric hepatitis phase with fever and nausea and proceed to hepatitis. Most HEV infections resolve after flu-like phase. Clinical signs include jaundice, anorexia, fever, abdominal tenderness, vomiting (149). Clinical signs in experimental animals like non-human primates are dose-dependent. Hepatitis E manifests as asymptomatic/subclinical, acute or fulminant type, but never proceeds to chronicity (38, 149). Fulminant type of hepatitis causes up to 1% mortality in general population but is rare in occurrence except in pregnancy with up to 15-25% mortality (38, 59, 76, 112).

**Diagnosis**

Diagnosis of HEV is important for proper supportive therapy as the symptoms of hepatitis E are indistinguishable from other disease conditions. Recombinant ORF2 and
ORF3 proteins expressed in various expression systems are widely used in Enzyme Immunoassays like ELISA and Western blot to detect IgG and IgM anti-HEV antibodies (5, 14, 17, 26, 139, 158). A positive test for anti-HEV IgM and a high or an increasing titer of anti-HEV IgG indicates an acute HEV infection. RT-PCR can detect HEV RNA in feces or serum and is used as a confirmatory test. Immune electron microscopy and immune fluorescence microscopy are not used routinely as they are expensive and laborious (149).

**Prevention and control**

Currently, commercial vaccines against HEV are not available. Passive immunoprophylaxis using antibodies to HEV capsid was successful in cynomolagus monkeys (138). Though there are 4 genotypes of HEV, the HEV strains share common epitopes on capsid gene suggesting that they belong to a single serotype (5, 120). Therefore, a protective vaccine against a broad spectrum of HEV isolates is possible. Lack of an efficient cell culture system hindered the development of killed or attenuated vaccines. The alternative strategy was to express recombinant proteins of HEV. Various recombinant capsid proteins expressed in insect cells and *E.coli* were reported to be successful recombinant vaccine conferring protection against both homologous and heterologous HEV strains (38, 39, 137).

HEV, an enterically transmitted virus, is primarily transmitted by contamination of drinking water and undercooked meat products. Personal hygiene, proper sanitation, consumption of clean water supplies and proper cooking of meat products will help prevent the disease.
**Hepatitis E as a zoonosis**

Anti-HEV antibodies have been reported in pigs both in the developing countries like China, India, Nepal and Thailand (10, 11, 32, 95, 146) and industrialized countries like United States, Canada, Japan, Australia, New Zealand, Taiwan and European countries (18, 19, 27, 31, 32, 44, 53, 95, 98, 100, 102, 107, 150) suggesting that HEV is enzootic in pigs worldwide. In 1997, a novel strain of HEV, designated as swine HEV, was identified from pigs in the United States (98). Since then, many HEV strains were identified from swine in various geographic regions of the world (10, 11, 18, 19, 27, 31, 32, 44, 63, 102, 128). Rats and rodents from United States were seropositive for anti-HEV antibodies (41, 61). Chickens were also found to be seropositive in Vietnam (135). A HEV-like virus was identified from chickens with big liver and spleen disease in Australia (105) and an avian strain of HEV from chickens with Hepatitis-Splenomegaly (HS) syndrome in North America (52). HEV seropositivity has been reported in domestic and feral animals of various species including sheep, cattle, goats, dogs and cats in both endemic and non-endemic areas (41, 61, 78, 92, 93, 103). Experimental HEV infections were demonstrated in pigs, laboratory rats and pigs (16, 84, 96, 144) and cross-species infections have been documented (50, 94, 97, 126). Serological and genetic identification of HEV strains from various species of animals, the HEV seroprevalence in swine veterinarians and swine handlers (92, 93, 99) and food-borne transmission of HEV by ingestion of undercooked meat products (87, 131, 133, 151) strongly indicate that animal reservoirs for HEV exist and that hepatitis E is a zoonotic disease.
Swine HEV

Balayan et al. in 1990 reported experimental infection of domestic pigs with human HEV isolate (16), followed by the detection of HEV RNA and anti-HEV antibodies in swine from Nepal by Clayson et al (32). However, the virus was not characterized. Meng et al. in 1997 discovered a novel strain of HEV, swine HEV in pigs of United States, and the genomic organization of swine HEV was identified to be very similar to human HEV (98). The two human HEV strains (US-1 and US-2) from U.S. patients with acute hepatitis were characterized (119). The USA strain of swine HEV was found to be closely related to HEV strains from USA (97). Swine HEV has been identified from pigs in many other countries and shown to be genetically closely-related to human HEV, especially the genotypes 3 and 4 strains of human HEV. Sequence and phylogenetic evidence indicates that the HEV isolates from humans and swine from same geographical area belong to same genotype (10, 11, 18, 19, 27, 31, 32, 44, 63, 102, 128). Interspecies transmission of swine HEV to non-human primates (97) and a US strain of human HEV to pigs have been documented (50). High prevalence of anti-HEV antibodies in humans in non-endemic areas is attributed to infection with swine HEV (38, 92). The zoonotic potential of HEV has implications for xenotransplantation involving pig liver, as the swine HEV may be inadvertently transmitted to the xenograft recipient.

Avian HEV

The first evidence of seropositivity to human HEV in chickens was observed in 44% of chickens in Vietnam, indicating that the chickens were exposed to HEV or a HEV-like agent (135). However, the causative agent was not isolated or characterized. In
1999, a big liver and spleen disease virus (BLSV) was identified from chicken in Australia (105). Sequence data based on a 523-bp sequence suggested that BLSV is genetically related to human HEV with a sequence identity of 62 % in the helicase region. Hepatitis-Splenomegaly (HS) syndrome, also called necrotic, hemorrhagic, hepatomegalic hepatitis (127) was first reported in 1991 in Canada (117). HS syndrome, an emerging chicken disease in North America including United States, is characterized by high mortality in commercial egg laying hens of 30 to 72 weeks of age with significant drop in egg production (20 %) (116, 117). Clinical features include ovarian regression, enlarged liver and spleen and red fluid in the abdomen. Though a viral etiology was suspected for HS syndrome, attempts to identify the viral agent were not successful (121).

In 2001, Haqshenas et al., isolated and characterized an avian strain of HEV, designated as avian HEV, from chickens with HS syndrome in United States (52). Electron microscopy revealed that avian HEV is a non-enveloped virus 30-35 nm in diameter and was reported to have similar size and morphological characteristics as human HEV (112). The complete genomic sequence of avian HEV was determined and found to be similar to that of mammalian HEVs (56). Inspite of an approximately only 50% nucleotide sequence identity with mammalian HEVs, avian HEV shares many significant structural and functional features with human and swine HEVs. Avian HEV also shares approximately 80% nucleotide sequence identity with the Australian chicken big liver and spleen disease virus (BLSV) (52, 105). Avian HEV was also reported to have common and unique antigenic epitopes in putative capsid protein with human and
swine HEV (49, 51). A recombinant avian HEV capsid protein was found to induce protective immunity against avian HEV infection (48).

Sequence analysis of a strain of avian HEV recovered from an outbreak of HS syndrome revealed sequence identities of 82-92% and 78-80% in helicase and capsid protein genes respectively compared to other avian HEV isolates (4). Phylogenetic analyses of avian HEV isolates identified in United States indicate that they belong to a new genotype (55). Avian HEV is similar to human HEV genetically and antigenically. Therefore, avian HEV-chicken model can be used to study pathogenesis and replication of HEV.

Recently it was reported that antibodies to avian HEV were also prevalent in healthy chicken flocks in the United States (55) suggesting that chickens in the United States are subclinically and chronically infected. From a recent prospective study, an apparently avirulent strain of avian HEV was isolated from healthy chickens without clinical disease (125). Chickens became infected with this apparently avirulent avian hepatitis E virus at about 3-4 months of age under natural conditions. However, the clinical signs of HS syndrome in infected chickens were absent. The nucleotide sequence identities in helicase and capsid genes between this apparently avirulent isolate and the pathogenic isolate obtained from chicken with HS syndrome were 75-97% based on a short sequence. Since the avian HEV strain from healthy chickens does not cause any clinical disease under field conditions, it is important to determine its complete genomic sequence and compare it to the pathogenic strain from chicken with HS syndrome. Thus far, the complete genomic sequence for avian HEV is reported only for a single pathogenic strain, the prototype avian HEV (56).
It is also important to investigate any differences in pathogenicity between the apparently avirulent strain and the prototype pathogenic strain, which may be related to the genetic differences by a comparative pathogenesis study.

**Animal models**

Macaques were the first experimental animals used in HEV studies. Though other non-human primates were used, macaques were the species of choice to study HEV (111). Cynomolgus macaques and rhesus monkeys were invaluable in studying the natural history of virus and for evaluation of candidate vaccines (7, 8, 38, 40, 138, 140, 156). However, due to the limited resources, ethical concerns and restricted experimental procedures available for the use of non-human primates, little has been learned about the pathogenesis of HEV using primate models. In addition, interpreting the significance of human HEV pathogenesis in non-human primates could be difficult as non-human primates are not the natural hosts of human HEV. With the discovery of swine HEV (98), swine HEV infection in pigs has been evaluated as an experimental model for HEV (50, 65, 147). However, the potential to use swine as a model system is limited by the fact that swine HEV causes only a subclinical infection(50, 96, 98). Therefore, only certain aspects of HEV replication and pathogenesis can be studied with the pig model. HEV is transmitted by fecal-oral route, but the experimental infections by oral route were not successful using non-human primates and pigs (64) and intravenous route is still the preferred route in experimental HEV infections (50, 96, 97, 141). The discovery of avian HEV and its association with a hepatic disease (52) provides a homologous model system
to study HEV pathogenesis and replication under the natural route of infection, the oral route.
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Chapter 2

Systematic pathogenesis and replication of avian hepatitis E virus in specific-pathogen-free adult chickens

Guenette, T.E. Toth, and X.J. Meng

ABSTRACT

Hepatitis E virus (HEV) is an important human pathogen. Due to the lack of a cell culture system and a practical animal model for HEV, little is known regarding its pathogenesis and replication. The discovery of a strain of HEV in chickens, designated avian HEV, prompted us to evaluate chickens as a model to study HEV. Eighty-five, 60-week-old, specific-pathogen-free chickens were randomly divided into three groups. Group 1 chickens (n=28) were each inoculated with $5 \times 10^5$ 50% chicken infectious dose of avian HEV by oronasal route, group 2 chickens (n=29) each with the same dose by the intravenous (IV) route, and group 3 chickens (n=28) uninoculated as controls. Two chickens from each group were necropsied at 1, 3, 5, 7, 10, 13, 16, 20, 24, 28, 35, and 42 days postinoculation (DPI) and the remaining ones at 56 DPI. Serum, fecal, and various tissue samples including liver and spleen were collected at each necropsy for pathological and virological testing. By 21 DPI, all oronasally- and IV- inoculated chickens had seroconverted to avian HEV antibodies. Fecal virus shedding was detected variably from 1 to 20 DPI in IV group, and 10 to 56 DPI in oronasal group. Avian HEV RNA was detected in serum, bile, and liver samples in both IV- and oronasally-inoculated chickens. Gross liver lesions, characterized by subcapsular hemorrhages or
enlargement of right intermediate lobe, were observed in 7/28 oronasally- and 7/29 IV-inoculated chickens. Microscopic liver lesions were mainly lymphocytic periphlebitis and phlebitis. The lesion scores were greater for oronasal (\(P = 0.0008\)) and IV (\(P = 0.0029\)) group birds than for control birds. Slight elevations of plasma liver enzyme lactate dehydrogenase were observed in infected chickens. The results indicated that chicken is a useful model for studying HEV replication and pathogenesis. This is the first report of HEV transmission via its natural route in a homologous animal model.
INTRODUCTION

Hepatitis E virus (HEV), the causative agent of hepatitis E, is responsible for the majority of enterically transmitted cases of non-A and non-B hepatitis (2, 34-35). HEV is a single-stranded, positive-sense nonenveloped RNA virus with a genome size of about 7.2 kb (34). The genome contains three open reading frames (ORFs) and short 5’ and 3’ non-translated regions. ORF1, the largest of the three, encodes nonstructural proteins, and ORF2 encodes the putative capsid protein. The small ORF3, which partially overlaps ORF1 and ORF2, encodes a cytoskeleton-associated phosphoprotein (34-35). Hepatitis E is epidemic and endemic in many developing countries. Sporadic cases of acute hepatitis E have also been reported in industrialized countries including the United States (25-27, 34, 38-39). The disease mostly occurs in young adults. Although the mortality rate is generally low, it can reach up to 25% in infected pregnant women (1, 22-23). The primary mode of HEV transmission is thought to be the fecal-oral route and waterborne epidemics is the most explosive form in developing countries of Asia and Africa (34-35).

Non-human primates have been used as animal models for HEV (3, 5, 10, 45, 48). However, due to the limited resources, ethical concerns and restricted experimental procedures available for the use of non-human primates, little has been learned about the pathogenesis of HEV using primate models. In addition, extrapolating from or interpreting the significance of human HEV pathogenesis in non-human primates could be difficult as non-human primates are not the natural hosts of human HEV. The first animal strain of HEV, swine HEV, was discovered in 1997 from a pig in the United States (30). Since then, swine HEV has been identified from pigs in many other countries and shown to be genetically closely-related to human HEV, especially to the genotypes 3
Interspecies transmission of swine HEV to non-human primates (29) and a US strain of human HEV to pigs (14) have been documented. Increasing evidence indicated that hepatitis E is a zoonotic disease (25, 27, 31). Swine HEV infection in pigs has been evaluated as an experimental model for HEV (14, 21, 46). However, the potential to use swine as a model system is limited by the fact that swine HEV causes only a subclinical infection (14, 28-29). Therefore, only certain aspects of HEV replication and pathogenesis can be studied with the pig model.

More recently, another animal strain of HEV, avian HEV, was identified and characterized from chickens with Hepatitis-Splenomegaly (HS) syndrome in United States (16). Like swine HEV, avian HEV is also genetically and antigenically related to human HEV. Unlike swine HEV, however, avian HEV is associated with a hepatic disease (HS syndrome) (16, 19). The complete genomic sequence of avian HEV was determined (20). The genomic organization of avian HEV is very similar to that of mammalian HEVs (20). Although avian HEV has only about 50% nucleotide sequence identity with mammalian HEVs, they share many significant structural and functional features (20), supporting the conclusion that avian HEV and mammalian HEVs belong to the same genus, *Hepevirus* (9). The discovery of avian HEV and its association with a hepatic disease provides a homologous animal model system to study HEV pathogenesis and replication. In this study, we attempted to experimentally infect specific-pathogen-free (SPF) adult chickens by the natural fecal-oral route, to systematically study HEV pathogenesis and replication in a homologous animal model via natural route of infection,
and to characterize the clinical course and pathological lesions associated with avian HEV infection.

**Attributions:** I acknowledge the attributions of the following people. F.F. Huang, Z.F. Sun, D.K. Guenette and F.W. Pierson for help with chicken experiment, R.B. Duncan for histopathological evaluation, F. Elvinger for statistical analysis and F.W. Pierson, T.E. Toth and X.J. Meng for intellectual help.

**MATERIALS AND METHODS**

**Virus.** The avian HEV used in this study was originally recovered from a bile sample of a 56-week-old chicken with HS syndrome (16). An infectious stock of avian HEV was generated and titrated in young SPF chickens (41). The avian HEV stock with an infectious titer of $5 \times 10^{4.5}$ 50% chicken infectious dose ($\text{CID}_{50}$) per ml was used as the inocula for this study.

**Chickens.** Eighty-five, 60-week-old, SPF chicken were purchased from Charles River SPAFAS Inc. (Wilmington, MA). The chickens were at the late stage of egg production. Prior to inoculation, all birds were confirmed to be negative for avian HEV antibodies by an enzyme-linked immunosorbent assay (ELISA) (19).

**Experimental design.** The chickens were randomly divided into 3 groups of 28, 29, and 28 chickens each. Twenty-eight chickens in group 1 were each inoculated by oro-nasal route with $5 \times 10^{4.5} \text{CID}_{50}$ of the avian HEV infectious stock. One-fourth of the 1 ml
inoculum was given nasally and the remaining inoculum was given orally by using gavage needles. Chickens in group 2 (n=29) were each intravenously (IV) inoculated with $5 \times 10^{4.5}$ CID$_{50}$ of the avian HEV infectious stock. Twenty-eight chickens in group 3 served as uninoculated controls. Each group was housed in a separate isolation room and the chickens were allowed access to feed and drinking water *ad libitum*.

**Sample collection and processing.** Blood and fecal swab materials were collected prior to inoculation and weekly thereafter. Weekly blood plasmas were tested for liver enzyme profiles. Weekly serum samples were tested by ELISA for anti-avian HEV antibodies. Weekly serum samples and fecal swab materials were tested for avian HEV RNA by RT-PCR. Two chickens from each group were necropsied at 1, 3, 5, 7, 10, 13, 16, 20, 24, 28, 35, and 42 days post-inoculation (DPI) and the remaining ones at 56 DPI. Samples of serum, feces, bile, and thirteen different tissues were collected during each necropsy and stored at $-80^0$ C. A portion of the liver tissue samples collected at each necropsy was homogenized in 10% (w/v) sterile phosphate-buffered saline (PBS). The liver homogenates were clarified by centrifugation at 3,000 rpm for 15 min at 4 $^0$C, and used for detection of avian HEV RNA by RT-PCR.

**Pathology and histopathology evaluations.** Gross pathological lesions were evaluated for liver and spleen during necropsies and also recorded as digital pictures. Tissue samples collected at each necropsy, including thymus, heart, liver, lung, spleen, kidney, colon, cecal tonsils, cecum, ileum, jejunum, pancreas and duodenum, were fixed in 10% neutral buffered formalin and processed for routine histological examinations.
Histopathological lesions in various tissues were evaluated in a blinded fashion by a veterinary pathologist (R.B.D) and were scored according to lesion severity based on standard scoring systems. Liver lesion scores range from 0 to 4 (0, no lesions; 1, < 5 foci; 2, 5-8 foci; 3, 9-15 foci; 4, >15 foci). Thymus lesions were given scores from 0 to 4 (0, none; 1, minimal; 2, mild; 3, moderate; 4, severe) based on the severity of the lesions. Lung lesion scores were expressed as numbers of foci. Kidney lesion scores range from 0 to 4 (0, no lesions/non-specific foci; 1, minimal interstitial nephritis; 2, mild interstitial nephritis; 3, moderate interstitial nephritis; 4, severe interstitial nephritis).

**Liver Enzyme Profiles:** A total of 13 chickens (4 from oronasal group, 5 from IV group, and 4 from control group) were monitored weekly throughout the entire study of 56 days. Serum levels of liver enzymes for the 13 chickens, including aspartate transferase (AST), lactate dehydrogenase (LDH), creatine phosphokinase, albumin/globulin (A/G) ratio, bile acids, and total proteins were determined by standard methods (Avian and Exotic Animal Clinical Pathology labs, Wilmington, OH).

**ELISA for avian HEV antibody.** A purified truncated recombinant ORF2 capsid protein of avian HEV expressed in *Escherichia coli* was used as the antigen for the ELISA to detect avian HEV antibodies in chickens as previously described (15, 19, 40). Briefly, the purified avian HEV antigen was coated onto 96-well plates (Thermo Labsystems, Franklin, MA). A horseradish peroxidase-conjugated rabbit anti-chicken IgG (Sigma Chemical Co., St. Louis, Mo.) was used as the secondary antibody. The optical density (OD) values were measured at 405 nm. Samples with OD values greater
than 0.30 were considered positive as determined previously (19, 40). Convalescent sera from experimentally infected chickens (41) and sera from SPF chickens were included as positive and negative controls, respectively.

**RT-PCR to detect avian HEV RNA.** To detect avian HEV RNA in feces, serum, bile, and liver tissue homogenates, RT-PCR was performed as previously described (19). Briefly, RNA was extracted with TriZol Reagent (GIBCO-BRL) from 100 µl of serum, 10% fecal suspension, 10% liver homogenate, or bile sample. Total RNA was resuspended in 12.25 µl of DNase, RNase, and proteinase-free water (Invitrogen). Reverse transcription was performed at 42°C for 60 min with 1 µl of N2 reverse primer (5’-CCGGGCTGATGGTCTCGATTAG-3’), 0.25 µl of Superscript II reverse transcriptase (Invitrogen), 1 µl of 0.1M dithiothreitol, 4 µl of 5x RT buffer, 0.5 µl of RNase inhibitor, and 1 µl of 10 mM dNTPs. Five microliters of the resulting cDNA was amplified in a 50 µl reaction using Amplitaq Gold DNA polymerase (Applied Biosystems).

For confirmation purpose, two nested RT-PCR assays targeted at different regions were used to test the samples. For the first nested RT-PCR assay, the first round PCR using a primer set located in the ORF1 region, forward primer N1 (5’-TTACCATTGACTTTGAACGGCG-3’) and the reverse primer N2 produced an expected fragment of 643 bp. In the second round nested PCR, the forward primer N3 (5’-GCTTGTGCATTGACGATTCCC-3’) and the reverse primer N4 (5’-CAATAGGTATTACCACGATGACG-3’) produced an expected fragment of 500 bp. For the second nested RT-PCR assay, the first round PCR produced an expected fragment of
595 bp with the forward primer P1 (5’- ACAACATCCACCCTACAAG-3’) and the reverse primer P2 (5’-ACAGTTTCACCTCAGGCTCG-3’). In the second round nested PCR, the forward primer P3 (5’-AGAACAAATGGTTGGCGGTCC-3’) and the reverse primer P4 (5’-GAGGGCAAGCCACCTAAAAC-3’) amplified an expected fragment of 394 bp. The PCR parameters included an initial incubation at 94°C for 9 min to activate AmpliTaq Gold DNA polymerase, followed by 39 cycles of denaturation at 94°C for 0.5 min, annealing at 58°C for 0.5 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min.

The PCR products amplified from serum, feces, bile and liver samples of 2 selected chickens from oronasally-inoculated group and 1 selected chicken from IV-inoculated group were sequenced to confirm the identity of the virus recovered from the experimentally infected chickens.

**Statistical analyses:** Gross and histopathologic lesions were recorded as either presence or absence of lesions, as lesion scores or counts of lesion foci. Categorical (dichotomous) variables were analyzed by logistic regression using either the LOGISTIC or the GENMOD procedures in SAS® (version 8.02; SAS Institute, Inc., Cary, N.C.). Lesion scores were ranked, and medians were compared by analysis of variance using the GLM procedure of SAS. Counts of lesion foci were modeled as either Poisson or Negative Binomial distributed variable using GENMOD procedure in SAS. Models included treatment (TRT) and DPI, and, in addition, for lesion scores included the TRT x DPI interaction.
RESULTS

Clinical signs. Clinical signs such as lowered feed consumption, diarrhea or mortality were not observed in any chickens of the three groups for the duration of the study.

Seroconversion to avian HEV antibodies in both oronasally- and intravenously-inoculated chickens. Prior to inoculation, all the chickens were seronegative. All control chickens were seronegative throughout the study. IgG anti-avian HEV were detected in 9 of 22 oronasally-inoculated and 10 of 23 IV-inoculated chickens at 1 WPI (Table 1). By 3 WPI, all remaining oronasally and IV-inoculated chickens had seroconverted. OD values differed between treatment groups ($P < 0.0001$) and behaved differently for each treatment group over the duration of the study ($P < 0.0001$; Fig. 1). Mean OD value for IV-inoculated chickens peaked at 2 WPI (OD Mean: $0.876 \pm 0.034$), decreasing by week 5 to a mean of $0.417 \pm 0.046$). Mean OD values for oronasally-inoculated chickens increased gradually up to 6 WPI (OD Mean: $0.778 \pm 0.056$), and then remained relatively stable.

Detection of avian HEV RNA in fecal, serum, bile, and liver tissue samples of both oronasally- and IV-inoculated chickens. Samples from all chickens were negative for avian HEV RNA at 0 DPI. All control chickens remained negative throughout the experiment. Avian HEV RNA was detected variably in the serum, fecal, bile, and liver tissue samples of chickens in both oronasal and IV groups (Table 2).

For those necropsied chickens, bile, serum, fecal, and liver samples were available for analyses (Table 2). Viremia was detected in sera of 6/8 IV group chickens
necropsied from 3 to 10 DPI and thereafter variably at 24 and 35 DPI. Fecal shedding of virus was detected in 16/16 chickens necropsied between 1 and 20 DPI, and none thereafter. Bile and liver samples were positive for avian HEV RNA in 14/14 chickens from 1 to 16 DPI, and intermittently positive thereafter. In oronasally-inoculated group, avian HEV RNA was first detected in feces and sera at 10 and 20 DPI, respectively. Fecal shedding of virus was detected in 17/20 of oronasally-inoculated chickens necropsied from 10 to 56 DPI. Bile and liver samples were positive for avian HEV RNA in 15/16 chickens necropsied from 3 to 24 DPI. Viremia was detected only in 1 of 2 chickens necropsied each at 20 and 35 DPIs.

Weekly fecal swab materials and serum samples from the inoculated chickens that were not necropsied were also tested for the presence of avian HEV RNA by RT-PCR (Table 3). Avian HEV RNA was detected variably in fecal swab materials of both oronasally- and IV-inoculated chickens (Table 3). Viremia was also detected variably from weekly serum samples (Table 3). For the thirteen chickens (4-ornasal, 5-IV, and 4-control) that were not necropsied until the end of the study, fecal shedding of virus and viremia were detected mostly in the first 2 weeks in IV-inoculated chickens (Table 4). In the chickens inoculated by oronasal route, avian HEV was shed in feces from 1 to 8 WPI. Viremia in this group lasted from 2 to 5 WPI.

Virus recovered from selected experimentally infected chickens in each group was sequenced for a 395 bp sequence in the ORF1 region and a 290 bp sequence in the ORF2 region. Sequence analyses confirmed that the virus recovered from experimentally infected chickens originated from the inoculum.
**Gross lesions:** Gross lesions were observed primarily in the liver. Subcapsular hemorrhages were noticed in 3/28 oronasally-inoculated chickens, one each necropsied at 5, 16, and 35 DPIs, and in 5/29 IV-inoculated chickens necropsied at 3, 5, 7, 16, and 24 DPIs (Fig. 2). Slightly enlarged right intermediate lobe of the liver was evident in 4/28 oronasally-inoculated chickens (one each necropsied at 5, 7, 20, and 42 DPIs), and in 2/29 IV group chickens (one each necropsied at 5 and 10 DPIs). Control chickens showed no gross hepatic lesions.

**Microscopic lesions.** The data on microscopic lesions in liver are summarized in Table 5. Lymphocytic periphlebitis and phlebitis foci were observed in liver sections of 28/28 oronasally-inoculated chickens and 28/28 IV-inoculated chickens but also in 22/27 control chickens (Figs. 3A, 3B). However, the histological liver lesions were mild in the control group, and moderate to severe in the oronasal and IV group chickens (Table 5). The severity of lesions peaked at 10 DPI within IV group. Hepatocellular necrotic foci were observed in 1/27 chickens in the control group, 1/28 in the oronasal group, and 3/28 chickens in the IV group (Fig. 3C). Other lesions such as amyloid-like foci containing amorphous hypocellular eosinophilic matrix (5/56) and subcapsular hemorrhages (4/56) were observed in the inoculated chickens (Figs. 3D, 3E). Overall histological liver lesion scores differed between treatment groups ($P = 0.0015$). They were greater for oronasal ($P = 0.0008$) and IV ($P = 0.0029$) group birds than for control birds (Least Squares Means (LSM)-Oronasal: 2.65; IV: 2.55; Control: 1.71; Standard error of means (SEM): 0.19). However, the mean scores did not behave differently over time (TRT x DPI: $P = 0.48$).
Lesions were also observed in spleen, thymus, kidney, and lung (Table 6). Mild lymphoid hyperplasia was found in spleen (Fig. 4). The spleen lesion scores differed between treatment groups ($P = 0.024$) and were higher for IV group chickens ($P = 0.0066$) than for control chickens (LSM-Oronasal: 2.06; IV: 2.31; Control: 1.81; SEM: 0.12). Spleen lesion scores did not differ over time. Mild cortical hypoplastic lesions were detected in thymus mostly towards the end of the study, but did not differ between treatment groups ($P = 0.17$). Lesions of occasional mild lymphocytic interstitial nephritis in kidneys were only detected sporadically from 7 oronasal, 11 IV, and 8 control group chickens, and the lesion scores did not differ between treatment groups ($P = 0.72$). Foci of mild lymphocytic and heterophilic parabronchial interstitial inflammation in the lung (Table 6) were noticed in 21/28 oronasal chickens, 21/29 IV chickens and 12/28 control chickens ($P = 0.103$). Counts of lymphocytic parabronchial inflammatory foci in the lungs differed between treatment groups ($P = 0.035$) with foci counts in IV group an average 131% higher ($P = 0.01$) and counts in oronasal group an average 72% higher ($P = 0.08$) than in control chickens. Microscopic lesions were absent in the tissues collected from gastroenteric tract except for lymphoid hyperplastic lesions in one chicken necropsied at 5 DPI, and serosal and mesenteric adenocarcinoma lesions in another chicken necropsied the same day, and both belonged to IV group (Table 6).

**Liver enzyme profiles.** Serum levels of liver enzymes AST, albumin/globulin (A/G) ratios and bile acids of 4 oronasal, 5 IV, and 4 control group chickens that were monitored during the entire study did not differ between treatment groups (data not shown). However, LDH levels behaved differently over time ($P = 0.0851$). In IV group,
LDH levels peaked at 1WPI and then returned to baseline levels. The LDH levels in oronasal group chickens remained elevated from 1 to 4 WPI, and 6 WPI prior to returning to baseline values at 7 WPI (Fig. 5). Total proteins (TP) differed between the inoculated groups ($P = 0.0634$) with the oronasally inoculated chickens having high TP than IV and control group chickens ($P < 0.0001$).

**DISCUSSION**

The main constraints for studying HEV are the lack of an *in vitro* cell culture system and of a practical animal model. Fecal-oral route is thought to be the natural route of HEV infection (26, 34-35, 46). Experimental infections by intravenous route of inoculation of HEV in swine and non-human primates (14, 21, 28-29, 45) have been well documented. However, under experimental conditions, infections of animals such as monkeys and pigs by HEV via the oral route of inoculation proved to be very difficult. Balayan et al (4) and Chauhan et al (7) transmitted hepatitis E to human volunteers by oral administration of pooled stool extracts from cases of non-A and non-B hepatitis. Gupta et al (13) observed biochemical, histopathological and serological changes in 2 monkeys orally-inoculated with pooled stool samples containing non-A, non-B hepatitis virus particle. However, others failed to experimentally infect animals via the oral route of inoculation, even with high titer of infectious HEV stock (21). Intravenous route is still the preferred route of inoculation in experimental HEV infections of swine and non-human primates (14, 28-29, 45). However, since IV route is not the natural route of transmission, studies for the pathogenesis and replication of HEV in IV-inoculated animals are limited. Currently, there is no report of experimental HEV infection in a homologous animal model system via the fecal-oral route.
The discovery of avian HEV in chickens with HS syndrome and the demonstrated antigenic and genetic relatedness between avian HEV and human HEV allowed us to use chickens as a homologous small animal model system to study HEV replication and pathogenesis. In the present study, we successfully infected 60-week-old SPF chickens with a strain of HEV from a chicken by the fecal-oral route, as well as the IV route. The course of pathogenesis and virus replication in chickens was characterized.

Under field conditions, HS syndrome is characterized by regressive ovaries, red fluid in abdomen, and enlarged liver and spleen (36-37). The avian HEV-infected chickens in this study exhibited mild gross pathological lesions characteristic of HS syndrome such as subcapsular hemorrhages and slight swelling of liver lobes (16, 19, 40), but the gross lesions were mild and limited to only a fourth of the infected chickens. Therefore, the gross lesions characteristic of HS syndrome such as enlargement of liver and spleen could not be consistently reproduced in experimentally infected SPF chickens. This is not surprising since our recent study showed that chickens from clinically healthy flocks are also infected by avian HEV (40). It is likely that avian HEV infection is an important but not the sole factor for the development of clinical HS syndrome. The microscopic liver lesions were mainly lymphocytic, heterophilic periphlebitis and phlebitis with occasional biliary vacuolation, amorphous hypocellular eosinophilic matrix, hemorrhages and necrotic foci. Such types of lesions in the liver were characteristics for HS syndrome, also called as necrotic, hemorrhagic, hepatomegalic hepatitis (42). The foci containing amorphous hypocellular eosinophilic matrix are possibly serum within ectatic vascular spaces and are similar to the changes described as amyloid-like materials by Tablante et al (42). However, Congo red staining revealed that
it was not amyloid. Mild lymphoplasmacytic heterophilic periphlebitic lesions were also observed in some seronegative control chickens. These mild liver lesions are considered normal background for older chickens. Lymphoplasmacytic inflammation and rare focal necrotic foci were also observed in control uninfected pigs and were considered to be normal background changes for pig livers (14). The mean scores of the histopathological liver lesions were statistically significant between either of the two inoculated groups and the negative control group, indicating that the liver lesions in the inoculated chickens are attributed to avian HEV infection.

Chickens inoculated by either oronasal or IV routes seroconverted to avian HEV antibodies, became viremic, and shed virus in feces. Avian HEV RNA was detected in bile and liver samples, indicating that the virus must have replicated in the liver. IgG anti-avian HEV appeared and peaked much earlier in IV-inoculated chickens (2-3 weeks) than in oronasally-inoculated chickens (4-6 weeks). This was anticipated since, in IV-inoculated chickens, avian HEV directly reached its target organ, liver, whereas in oronasally-inoculated chickens, the virus has to first replicate at primary sites before entering the blood stream and reaching the liver. Similar to this study, IgG anti-HEV was detected at 2.5 WPI in rhesus monkeys intravenously inoculated with a genotype 1 HEV (48). Also, SPF pigs intravenously inoculated with a US-2 strain of human HEV seroconverted at 2-3 WPI (14, 28). Clearly, the results from this study indicated that the timing for the development of IgG anti-HEV is related to the routes of inoculation. In a study on acute sporadic hepatitis E in Egyptian children, the IgG anti-HEV was reported to disappear within 6-12 months after infection (12). Similarly, the infected chickens in this study, especially the IV-infected ones, displayed a waning trend in the level of IgG
anti-HEV antibodies. This diminishing titer of IgG antibody was also reported in cases where acute and serial convalescent phase human or monkey sera were tested by immune electron microscopy (5, 32). Compared to the IV-inoculated chickens, the decrease in IgG anti-avian HEV antibody titers is less evident in the oronasally inoculated chickens. The pattern of antibody decay observed in oronasally-infected chickens likely represents the true pattern of natural HEV infection in humans.

Serum levels of liver enzymes including LDH, AST, CPK and bile acids, total protein, and A/G ratio were analyzed. No significant elevations of liver enzymes AST, CPK, or bile acids were observed. The LDH levels, indicative of recent damages to the liver and suggestive of an acute infection, peaked at 1 WPI (Fig. 5) in the oronasally-inoculated chickens, which corresponds to seroconversion to avian HEV antibody (Fig. 1). In the IV-inoculated group, the LDH levels peak at 1 WPI (Fig. 5) which preceded the highest titer of anti-avian HEV at 2 WPI (Fig.1) and severe histopathological lesions in liver (Table 5). LDH was reported to be the most sensitive indicator of liver cell damage based on tissue enzyme profile studies in racing pigeons (24). Increased LDH activities were observed in 33% of pigeons with aflatoxin B1-induced liver damage (6). It appears that LDH is also a good indicator for hepatic damage in chickens.

The disappearance of viremia corresponds to the rising titer of IgG anti-avian HEV. An oronasally-inoculated chicken (ID no. 4428, Table 4) shed the virus in feces for up to 8 WPI in the presence of IgG anti-avian HEV, suggesting the possibility of a persistent infection. This observation prompted us to sequence the virus recovered from this chicken at 8 WPI to determine whether the virus has undergone any mutations during
replication that would render it escape the neutralizing antibody. However, the sequence recovered from this infected chicken at 8 WPI was identical to that of the inoculum.

Overall, the time of seroconversion and detection of avian HEV in feces, serum, bile and liver occurred earlier in IV-inoculated chickens than in natural oronasally-infected chickens, suggesting that extrahepatic sites of replication exist under natural conditions. It has been suggested that hepatic damage in hepatitis E patients was caused by immune response to the invading virus, and not due to the direct replication of the virus in the liver (34). It is unclear as to how the virus reaches liver as HEV is transmitted by fecal-oral route. By using the pig model, Williams et al (2001) reported that HEV replicates extrahepatically. Replicative, negative strand HEV RNA was detected in small intestines, lymph nodes, and colon (46). Similarly, extrahepatic sites of replication were also reported in pigs naturally infected by swine HEV (8). Further studies are warranted to investigate the extrahepatic sites of avian HEV replication.

In summary, we successfully infected chickens with a strain of HEV via the natural route. To our knowledge, this is the first report of a successful experimental oral transmission of HEV in a homologous animal model. Although clinical signs and gross lesions characteristic of HS syndrome were not consistently reproduced, characteristic microscopic liver lesions consistent with HS syndrome were reproduced in both oronasally- and IV-infected birds. The oronasally- and IV-infected chickens developed an infection similar to that of human HEV infection in monkeys. The availability of a chicken model should help us further study the mechanisms of HEV replication and pathogenesis in the future.
ACKNOWLEDGEMENTS

We thank H. L. Shivaprasad and Peter Woolcock of University of California-Davis for providing the original bile sample containing avian HEV. This study was supported by grants from the National Institutes of Health (AI 01653, AI 46505, and AI 50611) and from the U. S. Department of Agriculture National Research Initiative Competitive Grant Program (NRI 35204-12531).

REFERENCES


Fig. 1. Courses of seroconversion to avian HEV antibodies in inoculated SPF chickens. The mean ELISA OD values of all the chickens from oral, IV, and control groups at each week post-inoculation are plotted.
Fig. 2. Gross lesion of a liver from an IV-inoculated chicken showing subcapsular hemorrhages (arrows).
Fig. 3. Microscopic lesions of the liver. (A). A liver section from an oronasally-inoculated chicken, showing lymphocytic and scattered heterophilic portal vein
periphlebitis. (B). A liver section from an IV-inoculated chicken showing focally intense lymphocytic venous phlebitis and periphlebitis. (C). A liver section from an IV-inoculated chicken showing locally extensive hepatocellular necrosis with lymphocytic inflammatory cell infiltration. (D). A liver section from an IV-inoculated chicken. Note architectural disruption and coalescing deposition of hypocellular homogenous eosinophilic matrix with displacement of hepatocellular cords. (E). A liver section from an oronasally-inoculated chicken. Note large focus of acute hemorrhage with local architectural disruption of hepatocellular cords and hepatic sinusoids. H&E staining.
Fig. 4. Microscopic lesions in the spleen from an IV-inoculated chicken. Note coalescing focus of lymphoid hyperplasia surrounding several ellipsoidal artery profiles. H&E staining.
Fig. 5. Serum level of liver enzyme Lactate dehydrogenase (LDH) in inoculated and control chickens. The mean LDH values of 13 chickens (4 oronasal, 5 IV, and 4 control chickens) that were monitored for the entire duration of the study at each week postinoculation (WPI) were plotted.
Table 1. Seroconversion to avian HEV antibodies in oronasally- and IV-inoculated chickens

<table>
<thead>
<tr>
<th>Group</th>
<th>Route of inoculation</th>
<th>No. seropositive / total tested at WPI&lt;sup&gt;a&lt;/sup&gt;:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oronasal</td>
<td>0/28 9/22 10/16 12/12 10/10 8/8 6/6 4/4 4/4</td>
</tr>
<tr>
<td>2</td>
<td>IV</td>
<td>0/29 10/23 17/17 13/13 11/11 9/9 6/7 3/5 3/5</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>0/28 0/22 0/16 0/12 0/10 0/8 0/6 0/4 0/4</td>
</tr>
</tbody>
</table>

<sup>a</sup>WPI, weeks postinoculation.
Table 2. Detection of avian HEV RNA in feces, serum, bile, and liver samples of chickens necropsied at different times

<table>
<thead>
<tr>
<th>Route of inoculation</th>
<th>Sample</th>
<th>No. positive / total no tested at DPI a:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Oronasal</td>
<td>Feces</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>0/2</td>
</tr>
<tr>
<td>IV</td>
<td>Feces</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>2/2</td>
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</tbody>
</table>

a Two chickens were necropsied at each DPI from 1 to 42 DPI. The remaining chickens (5, 4 and 4 chickens from IV, oronasal, and control groups, respectively) were necropsied at 56 DPI. No chicken was necropsied at 49 DPI. Samples from all chickens in control group remained negative throughout the experiment and was not included in the Table.
Table 3. Detection of avian HEV RNA in weekly sera and fecal swabs from chickens during the course of the study

<table>
<thead>
<tr>
<th>Group</th>
<th>Route of inoculation</th>
<th>No. positive in sera (fecal swab) / total no. tested at WPI&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Oronasal</td>
<td>0(0)/28</td>
</tr>
<tr>
<td>2</td>
<td>IV</td>
<td>0(0)/29</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>0(0)/28</td>
</tr>
</tbody>
</table>

<sup>a</sup>WPI, weeks postinoculation.
Table 4. Courses of viremia and fecal virus shedding in the 13 chickens that were not necropsied till the end of the study

Detection of viral RNA in fecal swab / serum samples at the following WPI\(^a\):

<table>
<thead>
<tr>
<th>ID No</th>
<th>Pre-inoculation</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tr>
<td><strong>Oronasally-inoculated Group</strong></td>
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<td>4406</td>
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<td><strong>Control Group</strong></td>
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<tr>
<td>4433</td>
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<td>+/-</td>
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<tr>
<td>4443</td>
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<td>+/-</td>
<td>+/-</td>
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<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
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<td>+/-</td>
</tr>
</tbody>
</table>

\(^a\)WPI, weeks postinoculation.
**Table 5.** Microscopic liver lesions in control, oronasal, and IV group chickens

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of chickens with lesions (mean score) at days postinoculation (DPI)(^a):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>2 (1.5)</td>
</tr>
<tr>
<td>Oronasal</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>IV</td>
<td>2 (2.5)</td>
</tr>
</tbody>
</table>

\(^a\) Two chickens were necropsied at each DPI from 1 to 42 DPI, and the remaining (5, 4 and 4 chickens from IV, oronasal, and control groups, respectively) were necropsied at 56 DPI. No chicken was necropsied at 49 DPI. The liver lesions included lymphoplasmacytic periphlebitis, phlebitis or necrotic lesions.

\(^b\) Only one chicken sample was available.
### Table 6. Presence of histopathological lesions in various tissues collected at necropsy

<table>
<thead>
<tr>
<th>Route of inoculation</th>
<th>Sample</th>
<th>No. of chickens with lesions at days postinoculation (DPI)(^b):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1  3  5  7  10 13 16 20 24 28 35 42 56</td>
</tr>
<tr>
<td>Thymus</td>
<td>Oronasal(^b)</td>
<td>0 0 1 0 0 2 0 1 1 2 2 2 3</td>
</tr>
<tr>
<td>Liver</td>
<td>0 0 2 0 2 2 2 2 2 2 2 2 2</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>0 0 2 0 2 2 2 2 2 2 2 2 2</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0 0 2 0 2 2 2 2 2 2 2 2 2</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0 0 2 0 2 2 2 2 2 2 2 2 2</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>IV</td>
<td>0 0 2 0 0 1 0 2 0 1 2 2 2</td>
</tr>
<tr>
<td>Liver</td>
<td>0 0 2 0 2 2 2 2 2 2 2 2 2</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>0 0 2 0 2 2 2 2 2 2 2 2 2</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>0 0 2 0 2 2 2 2 2 2 2 2 2</td>
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</tr>
<tr>
<td>Spleen</td>
<td>0 0 2 0 2 2 2 2 2 2 2 2 2</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0 0 2 0 2 2 2 2 2 2 2 2 2</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
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<td></td>
</tr>
<tr>
<td>Cec.tonsils</td>
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</tr>
<tr>
<td>Cecum</td>
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<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0</td>
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</tr>
<tr>
<td>Duodenum</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) No lesions were observed in colon, cecal tonsils, cecum, ileum, jejunum, pancreas, and duodenum at any DPI in the oronasal group. No lesions in the ileum and jejunum at any DPI in the IV group. No lesions in the colon, cecum, ileum, jejunum, and duodenum at any DPI in the control group. The tissues with no lesion were not listed in the Table.

\(b\) Two chickens were necropsied at each DPI from 1 to 42 DPI, and the remaining ones (5, 4 and 4 chickens from IV, oronasal, and control groups, respectively) were necropsied at 56 DPI. No chicken was necropsied at 49 DPI.

\(c\) Only one chicken was available for this particular tissue.
Chapter 3
Identification of Hepatitis E Virus Replication in Non-liver Tissues in a Chicken Model under Natural Route of Infection

P. Billam, F.W. Pierson, W. Li, T. LeRoith, R. B. Duncan, and X.J. Meng

To be submitted to Journal of Virology

ABSTRACT

Hepatitis E virus (HEV) is an important human pathogen. Due to the lack of a cell culture system and a practical animal model for HEV, little is known about its pathogenesis and replication. Our discovery of a strain of HEV in chickens, designated avian HEV, afforded us an opportunity to use chickens as a model to study HEV replication and pathogenesis. In a previous study, we successfully infected chickens (n=28) via its natural route of inoculation (orally) with avian HEV, whereas the control chickens (n=28) remained negative. The objective of the present study is to identify the extrahepatic sites of virus replication in chickens experimentally infected with avian HEV via its natural route of exposure. A variety of tissues including colorectum, jejunum, ileum, duodenum, cecum, cecal tonsils, thymus, spleen, lung, heart, kidney, pancreas and liver were collected at necropsies at 1, 3, 5, 7, 10, 13, 16, 20, 24, 28, 35, 42, and 56 days postinoculation (dpi). A negative strand-specific RT-PCR that can detect replicating HEV RNA was standardized and employed to identify the potential sites of HEV replication in various tissues. Although positive-strand HEV RNA was detected in all tissues at some point during the course of the experiment, even in the absence of viremia, the replicating negative-strand viral RNA was only detected in liver, serum,
colorectum, cecum, jejunum, ileum, duodenum and cecal tonsils, but not in other non-GIT tissues. Avian HEV capsid antigen was detected by immunohistochemical staining with avian HEV capsid-specific anti-peptide antibody in liver and GIT tissues including colorectum, jejunum, ileum, cecum, cecal tonsils and pancreas. The detection of avian HEV capsid antigen and replicative negative-strand viral RNA in the GIT tissues indicates that HEV replicates in the GI tract following infection by fecal-oral route. This is the first report of extrahepatic sites of HEV replication in experimental HEV infections via natural route of inoculation in a homologous animal model system.
INTRODUCTION

Hepatitis E virus (HEV) is a small, non-enveloped, positive-sense, single-strand RNA virus belonging to the genus Hepevirus in the family Hepeviridae (15). The genome size is 7.2 kb and contains 3 open reading frames (ORFs). ORF 1 codes for non-structural proteins like methyl transferase, papain-like cysteine protease, helicase and RNA-dependent RNA polymerase (RdRp). ORF2 encodes the capsid protein, and ORF3, the smallest of three ORFs is believed to code cytoskeleton-associated phosphoprotein and is also thought to have regulatory functions (16, 17, 53, 54). HEV does not grow well in cell culture (17) and the lack of an established in vitro cell culture system has been a major impediment in the study of HEV.

HEV causes hepatitis E in humans, which is a major public health concern in developing countries. HEV is responsible for major epidemics of acute hepatitis in countries of Asia, Africa and North America and sporadic cases of acute hepatitis in industrialized countries like United States, Japan and European countries (1, 2, 4, 17, 32, 36, 41, 53-56, 71, 74). Antibodies to HEV have been detected in a significant proportion of healthy individuals in industrialized countries like the United States though the incidence of disease is only sporadic in these countries (43, 44, 48, 64, 69). The urban sewage samples from non-endemic areas like France, Spain, Greece, Sweden and United States were tested positive for HEV RNA (14), suggesting that the healthy populations of the non-endemic areas may be exposed to animal strains of HEV that do not manifest any clinical symptoms. Hepatitis E is characterized by jaundice, malaise, nausea, vomiting, fever and abdominal tenderness (17, 70). Fecal-oral route is the primary mode of transmission (2, 4) and waterborne-epidemics caused by contamination of drinking water
supplies is characteristic of hepatitis E outbreaks (2, 53, 54). It mainly affects young adults and pregnant women, causing a 15-25% mortality rate in infected pregnant women (37).

In 1997, Meng et al (47) discovered the first animal strain of HEV, the swine HEV from commercial swine in the United States. Since then, many strains of swine HEV were isolated from pigs from different geographical regions of the world along with human strains (8-10, 12, 13, 20, 27, 28, 33, 39, 42, 46, 49-52, 59, 60, 63, 67, 72, 73). Subsequently an avian strain of HEV, designated as avian HEV, was isolated and characterized from chickens with Hepatitis-Splenomegaly (HS) syndrome in United States (26). The complete genomic sequence of avian HEV was determined and found to be similar to that of mammalian HEVs. Insipite of an approximately 50% nucleotide sequence identity with mammalian HEVs, avian HEV shares many significant structural and functional features with human and swine HEVs (30). Avian HEV also contains common and unique antigenic epitopes in its capsid protein with human and swine HEVs (21, 25), suggesting that avian HEV is similar to human HEV genetically and antigenically. The discovery of avian HEV and its association with a hepatic disease provided a homologous animal model system to study HEV pathogenesis and replication. The clinical and pathological findings associated with avian HEV infection in chickens under the natural route of infection have been reported (6). More recently, an apparently avirulent strain of avian HEV was isolated from healthy chickens without clinical disease (61) and its complete sequence was determined (7).

Due to lack of an efficient cell culture system and a practical animal model, very little is known about the pathogenesis of HEV. It has been suggested that HEV may
replicate in tissues other than liver (3). Evidence from experimental HEV infection in rats suggested that HEV may replicate extrahepatically (40). Extrahepatic sites of HEV replication were demonstrated in pigs experimentally infected via the intravenous route of inoculation (68) but reproduction of HEV infection via the natural oral route of inoculation in pigs and non-human primates has been very difficult. The objective of this study is to utilize the unique avian HEV infection in chicken homologous model system that can be easily reproduced under natural route of inoculation for definitive identification of the extrahepatic sites of HEV replication.

**Attributions:** I acknowledge the attributions of the following people. T. LeRoith and W. Li for immunohistochemistry, F.W. Pierson, R.B. Duncan and X.J. Meng for intellectual help.

**MATERIALS AND METHODS**

**The Virus**

The avian HEV used in this study was originally recovered from a bile sample of a 56-week-old chicken with HS syndrome (26). An infectious stock of avian HEV was generated and titrated in young SPF chickens (62). The avian HEV stock with an infectious titer of $5 \times 10^{4.5}$ 50% chicken infectious dose (CID$_{50}$) per ml was used as the inocula for the animal study.

**Experimental infection of chickens**

The experimental infection of SPF chickens with avian HEV and the clinical and pathological findings have been previously reported (6). Briefly, eighty-five, 60-week-
old, specific-pathogen-free (SPF) chickens were randomly divided into three groups. Group 1 chickens (n=28) were each inoculated with $5 \times 10^5$ 50% chicken infectious dose of avian HEV by natural oronasal route. Group 2 chickens (n=29) were each given the same dose by the intravenous (IV) route. Chickens in group 3 (n=28) were uninoculated controls. Two chickens from each group were necropsied at 1, 3, 5, 7, 10, 13, 16, 20, 24, 28, 35, and 42 days postinoculation (DPI) and the remaining ones at 56 DPI. Thirteen different tissues including colorectum, jejunum, ileum, duodenum, cecum, cecal tonsils, thymus, spleen, lung, heart, kidney, pancreas and liver were collected at necropsy. The tissues specimens were stored at -80°C until use. Tissue specimens from the oronasally inoculated chickens (Group 1) were used in the present study.
**Tissue homogenates**

Samples of tissues collected at necropsy from the oronasal group as well as the control group were homogenized in 10% sterile phosphate-buffered saline and stored at -80°C. At the time of RNA extraction, a portion of the tissue homogenate was clarified by centrifugation at 3,000 rpm for 15 min at 4°C (Eppendorf centrifuge 5810, rotor A-4-44) and subsequently used for the detection of avian HEV RNA by RT-PCR.

**RT-PCR to detect avian HEV RNA.** RT-PCR was performed to detect avian HEV RNAs in tissue homogenates as previously described (29). Briefly, RNAs were extracted by the use of TRI reagent (MRC) from 100 µl of the clarified 10 % tissue homogenate. Total RNA was resuspended in 12.25 µl of DNase-, RNase-, and proteinase-free water (Invitrogen). Reverse transcription was performed at 42°C for 60 min with 1 µl [10 pM] of P2 reverse primer (5’-ACAGTTTCACCTCAGGCTCG-3’), 0.25 µl [50 units] of Superscript II reverse transcriptase (Invitrogen), 1 µl of 0.1 M dithiothreitol, 4 µl of 5 X RT buffer, 0.5 µl [10-20 units] of RNasin ribonuclease inhibitor (Promega), and 1 µl of 10 mM deoxynucleoside triphosphates. Five microliters of the resulting cDNA was amplified in a 50 µl reaction with Platinum High Fidelity Supermix (Invitrogen) by using a nested PCR. The first round PCR produced an expected fragment of 595 bp with the forward primer P1 (5’- ACAACATCCACCCCTACAAG-3’) and the reverse primer P2. For the second round PCR, the forward primer P3 (5’-AGAACAATGGTTGGCGGTCC-3’) and the reverse primer P4 (5’-GAGGGCAAGCCACCTAAAAC-3’) amplified an expected fragment of 394 bp. The PCR parameters consisted of an initial incubation at 94°C for 9 min, followed by 39 cycles of denaturation at 94°C for 0.5 min, annealing at
52°C for 0.5 min, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR conditions for 2nd round PCR is the same as that of 1st round except for an annealing temperature of 56°C. For the detection of replicative negative strand RNA, reverse transcription was performed as previously described using EF1 primer (5’-ATGTTGGTGGGTGCTGGTCGAGATTG-3’). The primers EF1 and ER1 (5’-GGGTTGATTGGGCTCCGATATGATGCCAG-3’) were used in 1st round and EF2 (5’-TTGTTGGACATAACCCCGGCCCAC-3’) and ER2 (5’-TAATCACCAGAGACGGCTAGTGG-3’) were used in the 2nd round of nested PCR for the detection of negative strand RNA.

**Generation of a synthetic negative-strand avian HEV RNA as a positive control for demonstration of virus replication.** To standardize a negative strand-specific RT-PCR, a negative strand of HEV RNA had to be generated for use as a positive control. Briefly, total RNA was extracted with TRI reagent (MRC) from feces of a SPF chicken experimentally infected with avian HEV. Reverse transcription was performed at 42°C for 60 min with 1 µl [10 pM] of N2 reverse primer (5’-CCGGGCTGATGGTCTCGATTAG-3), 0.25 µl [50 units] of superscript II reverse transcriptase (Invitrogen), 1 µl of 0.1M dithiothreitol, 4 µl of 5x RT buffer, 0.5 µl [20 units] of RNasin RNase inhibitor (Promega), and 1 µl of 10 mM dNTPs. Five microliters of the resulting cDNA was amplified in a 50 µl reaction using Platinum High Fidelity Supermix (Invitrogen). A 494 bp ORF 1 fragment of avian HEV was amplified by PCR using primers F3088 (5’-CGCTGTAGTGAGCCATGTGTGGTG-3’) and primer R3559 (5’-TGTCTCGAGGGGTTGATTGGTC-3’). The PCR parameters consisted of
an initial incubation at 94°C for 9 min, followed by 39 cycles of denaturation at 94°C for 0.5 min, annealing at 58°C for 0.5 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The forward primer F3088 has an introduced BamHI restriction site and reverse primer R3559 has an XhoI to facilitate the subsequent cloning steps. The resulting PCR product was excised from the agarose gel and purified using GENE CLEAN II Kit. The purified PCR product was ligated into a TA vector using T4 DNA ligase (Stratagene). The ligation mixture was transformed into One Shot® TOP 10 chemically competent E. coli cells (Invitrogen) and 100 µl was spread on LB agar plates containing ampicillin and incubated overnight. After identification of plasmid containing insert and confirmation by restriction digestion, the insert was subcloned into pBluescript II SK (+) plasmid (Stratagene) by directional cloning using the restriction enzymes BamHI and XhoI. The recombinant PSK II plasmid containing ORF 1 insert was isolated and confirmed by sequencing. The recombinant PSK II plasmid was linearized and purified by phenol-chloroform extraction and a synthetic negative strand RNA was transcribed in vitro using mMMESSAGE mMACHINE® T7 kit (Ambion) transcription kit. To remove plasmid DNA, the transcribed negative-strand RNA was separated in a 1 % agarose gel, and the RNA band was excised from the gel and purified using RNaid isolation kit (Q biogene). The purified RNA was further treated with DNase for 60 min at 37°C. The RNA was then extracted with TRI reagent to further eliminate plasmid contamination. A nested PCR was done using two sets of primers (EF1, ER1, and EF2 and ER2) to rule out any potential plasmid contamination.
Standardization of a negative strand-specific RT-PCR assay. The \textit{in vitro} transcribed synthetic negative-strand RNA was quantified and serially diluted from 100 ng to $10^{-15}$ ng in 100\(\mu\)l PBS, and the negative strand-specific RT-PCR was performed on each serial dilution. Reverse transcription was performed with forward primer EF1 and nested PCR using E1 and F1 in 1\textsuperscript{st} round and EF2 and ER2 in 2\textsuperscript{nd} round, as previously described. The 232 bp product obtained from the second round of the nested PCR was sequenced for confirmation. Subsequently, the tissues that tested positive for positive-strand viral RNA were re-tested by the negative strand-specific RT-PCR to detect the replicating negative-strand viral RNA.

**DNA sequencing.** The PCR products were excised from 0.8\% agarose gel, purified using Geneclean III kit (Qbiogene), and sequenced for both strands at the Virginia Bioinformatics Institute Core Laboratory Facility using an automated DNA sequencer (Applied Biosystems).

**Production of avian HEV ORF2-specific anti-peptide antibody.** The ORF2 capsid protein of avian HEV was analyzed to select an antigenic peptide, acetyl-TKATVGVQVKVDAC-amide. The peptide was chemically synthesized and anti-peptide rabbit antibody specific for avian HEV ORF2 capsid antigen were produced both at 21st Century Biochemical Inc. as a custom antibody production service.

**Immunohistochemistry (IHC).** The IHC was done by Avidin Biotin Complex (ABC method (23) using the anti-peptide antibody specific for avian HEV ORF2 capsid after
standardization with varying dilutions of the primary antipeptide antibody. Briefly, the paraffin sections were incubated at 56°C for 30 min and then deparaffinized with 3 changes of xylene followed by rehydration with graded alcohols. The rehydrated sections were incubated in 1% hydrogen peroxide in methanol for 30 min to minimize non-specific peroxidase activity, followed by blocking with pre-diluted normal goat serum (1.5%) in PBS for 20 min and overnight incubation with the primary anti-peptide rabbit antibody diluted in blocking serum (1:300) at 4°C. After wash with PBS buffer, the sections were incubated with the secondary biotinylated anti-rabbit IgG antibody diluted in PBS (1:200) (Vector labs), washed with PBS buffer, followed by incubation with ABC reagent (Vector labs) for 5 min and DAB substrate (diamino benzidine) (Vector labs) for 10 min in dark, with a PBS wash step between ABC and DAB incubations. The slides were then washed with water and counterstained with hematoxylin, followed by dehydration in graded alcohols and xylene. The slides were air dried, and mounted with permount (Fisher). Sections from tissues of uninoculated control group chickens were used as negative controls. IHC was performed on tissues including liver, pancreas, colorectum, jejunum, ileum, cecum, cecal tonsils and duodenum from the oronasally-inoculated chickens.

RESULTS

Distribution of positive-strand avian HEV RNA in tissues. Positive-strand avian HEV RNA was detected variably from 1 to 56 dpi in various tissues. However, it was mostly detected in the liver and gastrointestinal tract (GIT) tissues and was rarely seen in non-GIT tissues (Table 1). Viremia was transient at 20 and 35 dpi (6) and viral RNA was
detected in most of the tissues at that time. Positive-strand viral RNA was detected in a number of tissues even in the absence of viremia. Liver was positive for the viral RNA from 3 dpi (6). Avian HEV RNA was detected in colorectum consistently from 1 dpi to 24 dpi and then at 35 and 56 dpi. Jejunum was positive from 7 dpi and ileum from 1 dpi. Viral RNA was detected in duodenum only from approximately third week but was consistently detected in cecum almost during the entire duration of the experiment. Cecal tonsils were transiently positive. Positive-strand viral RNA was also detected in thymus, spleen, lung, heart, kidney and pancreas. All control chickens were seronegative throughout the study, and they were not tested by RT-PCR or immunohistochemistry.

**Standardization of a negative-strand-specific RT-PCR assay.** Using a synthetic negative-strand viral RNA that was transcribed *in vitro* as a positive control, a negative-strand-specific RT-PCR assay was standardized. The 232 bp product from the second round PCR was sequenced to confirm the specificity. The negative-strand RT-PCR assay can detect up to a dilution level of 10 pg in first round and $10^{-5}$ pg in second round of nested PCR (Fig.1), corresponding to a detection level of approximately 3.75 million and 37.5 copies of viral RNA in first round and second round respectively.

**Detection of replicating negative-strand viral RNA in extrahepatic tissues.** The standardized negative-strand-specific RT-PCR assay was used to re-test all the tissues that were positive for the positive-strand viral RNA. Replicative negative-strand viral RNA was detected in the liver as well as in the GIT tissues but not in thymus, spleen, lung, heart, kidney or pancreas (Table 2). Replicative negative-strand viral RNA was
detected from 5 to 56 dpi in the GIT tissues. Liver was positive from 16 dpi. The earliest appearance of replicative negative-strand viral RNA was found in colorectum tissues at 5 dpi and in ileum at 7 dpi. Most of the GIT tissues were positive for replicative viral RNA at 20 and 35 dpi, the time point when viremia was detected. Cecal tonsil was positive for replicative viral RNA even at 56 dpi (Table 2).

Detection of avian HEV-specific capsid antigen in extrahepatic tissues by immunohistochemistry (IHC). Immunohistochemical staining of oronasally-inoculated chickens revealed positive signal for avian HEV capsid protein in the liver and most of the GIT tissues that were tested (Table 3). Areas of intense positive signal in the liver were visible as multiple foci of hepatocyte aggregates, characterized by cytoplasmic reactivity in the hepatocytes. The positive signal in some cases was found to be associated with the presence of lymphocytes, indicating an active inflammation (Fig.2A, 2B). Positive signal was also detected in colorectum and jejunum at all dpi (except 5dpi for colorectum). Focal areas of strong cytoplasmic reactivity were observed in the lamina propria and mucosal epithelium of the villi in the GIT tissues (Fig. 3A, 3B). Positive IHC signal was also detected in 21/28 and 14/28 of jejunum and colorectum sections, respectively, and in 10/28 of ileum and cecum sections. Duodenum, cecal tonsils and pancreas were also positive for avian capsid protein. No IHC signal was observed in the sections from uninoculated control group that were used as controls.
DISCUSSION

Knowledge regarding HEV pathogenesis and replication is limited due to lack of an efficient cell culture system and a practical animal model. Most of the scant knowledge about HEV pathogenesis was obtained from non-human primates (38, 46, 65, 66). However, the scarcity of resources, ethical concerns and the limited procedures that can be performed on non-human primates limit their use for HEV pathogenesis study. With the discovery of swine HEV (47), a swine model has been developed to study HEV (5, 18, 24, 31, 35, 45, 46, 68). Unlike non-human primates, swine are the natural hosts of swine HEV. However, swine HEV only causes a subclinical infection (24, 45, 46) and is not suitable to study all aspects of HEV especially HEV pathogenesis. More recently, another animal strain of HEV, avian HEV, was identified and characterized from chickens with HS syndrome (26). Avian HEV is antigenetically and genetically similar to human HEV (26, 30). HEV is transmitted by fecal-oral route (53), but the experimental infections by oral route were not successful using non-human primates or pigs (34) and intravenous route is still the preferred route in experimental HEV infections (24, 45, 46, 66). The similarity of avian HEV to human HEV and its association with a hepatic disease (HS syndrome) provides an inexpensive, homologous, small animal model system to study HEV pathogenesis. Most importantly, chickens can be easily infected via the oral route of inoculation with avian HEV (6) modeling precisely the situation in human. In the present study, tissues from 28 oronasally-infected chickens from a previous study (6) were investigated to identify extrahepatic sites of HEV replication in a homologous model under natural route of infection.
The mechanism of hepatic damage by HEV is not known. It has been suggested that the liver damage is caused by the immune response to the invading virus, and not by the direct replication of the virus in the liver (53). It is unclear how the virus reaches the liver, as HEV is transmitted by fecal-oral route. It is believed that the virus first replicates in the gastrointestinal tract (GIT) following oral ingestion and then reaches the target organ, liver. Extrahepatic sites of replication were demonstrated in a pigs by experimental intravenous inoculation of pigs with swine HEV and human HEV (68). Replicative, negative-strand HEV RNA was detected in small intestine, lymph node and colorectum in IV-infected pigs. However, the IV route of inoculation limited the ability to study the initial sites of virus replication following natural exposure. In this study, we identified the sites of extrahepatic HEV replication by employing a negative-strand-specific RT-PCR assay and immunohistochemistry in a homologous model system under natural route of infection.

The tissues collected from chickens oronasally-infected with avian HEV were first tested by RT-PCR to detect positive-strand viral RNA. All the tissues were positive for viral RNA at some point during the course of study. The tissue distribution of positive-strand HEV RNA was prominent in the liver and GIT tissues, but rare in non-GIT tissues like thymus, spleen, kidney, lungs and heart, suggesting that the liver and GIT tissues may be the sites of virus replication. The detection of positive-strand viral RNA in tissues does not necessarily mean that virus replicates in the tissues and may be due to circulating virus via viremia. However, positive-strand viral RNA was also detected in some tissues in the absence of viremia, thus suggesting that the positive-strand RNA in these tissues is likely the result of replication and not the circulating virus.
Colorectum, ileum, cecum, cecal tonsils are positive from as early as 1 dpi, suggesting that they are likely the initial sites of HEV replication after oral inoculation of the virus. The occasional detection of positive-strand viral RNA in non-GIT tissues could be due to the circulating virus. To further confirm the sites of extrahepatic HEV replications, we attempted to detect replicative negative strand viral RNA as well as virus-specific antigen in these tissues.

HEV, being a single-strand positive-sense RNA virus, produces an intermediate negative-strand viral RNA during replication. Therefore, detection of negative-strand viral RNA in tissues would confirm virus replication. A standardized negative-strand-specific RT-PCR was performed on all the tissues containing positive-strand viral RNA to detect the replicative negative-strand viral RNA. The results showed that replicative viral RNA was detected only in liver and GIT tissues including colorectum, jejunum, ileum, duodenum, cecum and cecal tonsils, but not in non-GIT tissues, thus strongly indicating that the GI tissues are the extrahepatic sites of HEV replication. It is interesting to note that the lymphoid tissues associated with the GIT are also positive for replicative viral RNA but not thymus. From the fact that colorectum was positive for replicative viral RNA from as early as 5 dpi and ileum from 7 dpi, while liver was positive from 16 dpi, it can be concluded that HEV initially replicates in the GIT tissues following fecal-oral transmission, and then reaches the target organ, liver.

Immunohistochemistry of liver and GIT tissues revealed positive IHC signal in a variety of tissues at different dpi’s (Table 3). The reactivity to avian HEV capsid protein was more pronounced in GIT tissues from 10 dpi to the end of the study at 56 dpi. In contrast to the evidence of positive hybridization and IHC signal in only lamina propria
of intestines of naturally infected pigs (11, 22), the signal was present in the cytoplasm of mucosal epithelium, apart from lamina propria of the GIT tissues in experimentally-infected chickens (Fig3A and 3B). Positive IHC reactivity was consistently observed in colorectum and jejunum indicating that they are the primary sites of extrahepatic replication. The cytoplasmic reactivity of hepatocytes in liver is in agreement with similar findings in the same study involving pigs naturally-infected with swine HEV (22). Most importantly, the positive IHC signal in liver was associated with periphlebitis, one of the characteristic lesions of experimental avian HEV infection by natural route of inoculation (6). The tissues were positive by immunohistochemistry earlier than by negative-strand specific RT-PCR suggesting that the negative-strand specific RT-PCR assay was less sensitive compared to immunohistochemistry possibly due to the presence of PCR inhibitors and the tissue homogenates used in the assay may not be perfectly representative of the whole tissue. The serum was also tested by negative-strand specific RT-PCR and interestingly, replicative viral RNA was detected at 20 and 35 dpi, corresponding to viremia and coinciding with the time point where the positive IHC signal and presence of replicative viral RNA was seen in most of the GIT tissues, indicating the replicative viral RNA in serum may be due to an overflow from the peak level replication going on in many GIT tissues at the same time. Similar to HEV, Hepatitis C virus (HCV) is a single-stranded positive-sense RNA and produces an intermediate replicative negative strand during replication. The detection of replicative viral RNA in sera could also be due to the circulating cell-associated viruses during viremia period. Negative strand viral RNA of hepatitis C virus (HCV) have also been
reported in sera of patients with chronic hepatitis C and in experimentally infected chimpanzees with acute HCV infection by strand-specific RT-PCR (19, 57, 58).

In summary, we successfully identified the extrahepatic sites of HEV replication in a homologous chicken model under the natural route of virus infection. We showed that viral capsid protein as well as replicative viral RNA can be detected in liver and GIT tissues and that the GIT tissues are initial sites of viral replication followed by liver as target organ. The biological and pathological significance of these extrahepatic replication sites is still not known but it may aid in the development of an \textit{in vitro} cell culture system for HEV.

\section*{ACKNOWLEDGEMENTS}

We would like to thank Oscar Peralta and Kathy Lowe for their help with immunohistochemistry. This study was supported by grants from the National Institutes of Health (AI 01653, AI 46505, and AI 50611) and from the U. S. Department of Agriculture National Research Initiative Competitive Grant Program (NRI 35204-12531). We thank Dr. Thomas Toth and Dr. Jake Tu for their support.
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(avian HEV) that are common to human and swine HEVs or unique to avian HEV. J Gen Virol 87:217-23.


Fig. 1. Standardization of negative-strand-specific RT-PCR. The synthetic HEV RNA transcript was serially diluted and tested by nested PCR. The expected PCR products in the first and second rounds are shown. M, 1 kb plus ladder.
Fig. 2

(A) Section of liver from a chicken oronasally inoculated with avian HEV. Two foci of centrilobular heptocytes have diffuse positive cytoplasmic reactivity using an anti-peptide antibody against avian HEV capsid protein. The positive cytoplasmic signal is associated with a small focus of lymphocytic perihlebitis. (B) Section of liver from a chicken oronasally inoculated with avian HEV. In one focus, heptocytes have diffuse positive cytoplasmic reactivity using an anti-peptide antibody against avian HEV capsid protein. The positive cytoplasmic signal is associated with a small aggregate of lymphocytes. The sections were counterstained with haematoxylin.
Fig. 3. (A) Section of colorectum from a chicken oronasally inoculated with avian HEV. There is diffuse cytoplasmic staining for avian HEV within the mucosal epithelium of several villi. Additionally, the underlying lamina propria is positive for avian HEV capsid protein. (B) Section of jejunum from a chicken oronasally inoculated with avian HEV. A focal area of mucosal epithelium has diffuse positive cytoplasmic reactivity using an anti-peptide antibody against avian HEV capsid protein. The sections were counterstained with haematoxylin.
Table 1. Detection of positive-strand HEV RNA in necropsy tissues of oronasally inoculated chickens.

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<tr>
<td>Colorectum</td>
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*Tissue specimens at 13 dpi were not tested.
Table 2. Detection of replicative negative-strand HEV RNA in necropsy tissues of oronasally inoculated chickens.

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^*Tissue specimens at 13 dpi were not tested.

NT: Not tested
Table 3. Immunohistochemistry of tissues from oronasally inoculated chickens

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*One sample was not tested in pancreas at 28 dpi and in cecal tonsils at 56 dpi.
Chapter 4

Analysis of the complete genomic sequence of an apparently avirulent strain of avian hepatitis E virus (avian HEV) identified major genetic differences compared to the prototype pathogenic strain of avian HEV

P. Billam, Z. F. Sun, and X. J. Meng

J Gen Virol; 88: 1538-1544

ABSTRACT

Avian hepatitis E virus (avian HEV) was identified from chickens with hepatitis-splenomegaly syndrome and is genetically and antigenically related to human HEV. Recently it was found that healthy chickens from some farms were also infected presumably by an avirulent strain. In this study, the complete genomic sequence of an avirulent strain of avian HEV was determined using primers based upon the prototype pathogenic avian HEV. The full-length genome of the avirulent strain, excluding the poly(A) tail, is 6649 nts in length, 5 nts shorter than the prototype avian HEV. Sequence analyses revealed that the ORF1 has a nucleotide sequence identity of 89.6% with numerous non-silent mutations and deletions compared to the prototype avian HEV. ORF2, which codes for the capsid gene showed a sequence identity of 90.7%, and six non-silent mutations (C4R, A288T, M359L, S436A, S511N, and R600K) were identified in this region. The ORF3 had four non-silent mutations with 97% sequence identity. Overall the avirulent strain has a nucleotide sequence identity of 90.1% with the prototype pathogenic strain. Phylogenetic analysis revealed that the avirulent strain is clustered together with the pathogenic avian HEV and represents a branch distinct from
mammalian HEVs. The identification of significant non-silent mutations in the capsid gene and other coding regions suggests that these mutations may play a role in HEV attenuation. This is the first report of the full-length genomic sequence of an avirulent strain of HEV, and is only the second strain of avian HEV to be fully sequenced thus far.
INTRODUCTION

Hepatitis E virus (HEV) is responsible for the majority of enterically transmitted cases of non-A and non-B hepatitis in humans (Arankalle et al., 1994; Purcell & Emerson, 2001). Hepatitis E is an important human disease of public health importance in many developing countries of the world. Sporadic cases of acute hepatitis E have also been reported in industrialized countries including the United States (Emerson & Purcell, 2004; Harrison, 1999; Meng, 2000, 2003, 2005; Purcell & Emerson, 2001; Schlauder et al., 1998; Schlauder & Mushahwar, 2001; Wang et al., 2001). The disease mostly occurs in young adults and the associated mortality is generally low, but can reach up to 25% in infected pregnant women (Aggarwal & Krawczynski, 2000; Khuroo & Kamili, 2003). HEV is primarily transmitted by fecal-oral route and waterborne epidemics are characteristic of the hepatitis E outbreaks in developing countries of Asia and Africa (Emerson & Purcell, 2003; Harrison, 1996; Purcell & Emerson, 2001; Sun et al., 2004). Recently, zoonotic food-borne transmissions of HEV from wild deers, wild boars and domestic pigs have been reported in Japan (Matsuda et al., 2003; Tamada et al., 2004; Tei et al., 2004; Tei et al., 2003).

HEV is a single-stranded, positive-sense non-enveloped RNA virus belonging to the genus Hepevirus (Emerson et al., 2004). The genome size is 7.2 kb and has three open reading frames (ORFs) (Emerson et al., 2001; Purcell & Emerson, 2001; Reyes, 1997). The largest of the three ORFs is ORF1 which codes for the nonstructural proteins including the methyltransferase, protease, helicase and RNA-dependent RNA polymerase (RdRp), while ORF2 codes for the capsid protein. The small ORF3 encodes a cytoskeleton-associated phosphoprotein (Purcell & Emerson, 2001).
The first animal strain of HEV, swine hepatitis E virus (swine HEV), was isolated from a pig in USA in 1997 (Meng et al., 1997). Since then many strains of swine HEV were isolated from pigs in more than a dozen countries worldwide and were shown to be closely related to the human HEV strains (Garkavenko et al., 2001; Haqshenas & Meng, 2001; Hsieh et al., 1999; Huang et al., 2002a; Nishizawa et al., 2003; Okamoto et al., 2003; Okamoto et al., 2001; Takahashi et al., 2003a; Takahashi et al., 2003b; van der Poel et al., 2001; Wang et al., 2002; Williams et al., 2001). More recently, another animal strain of HEV, avian HEV, was identified and characterized from chickens with hepatitis-splenomegaly (HS) syndrome in the United States (Haqshenas et al., 2001). Avian HEV is genetically and antigenically related to human HEV, and is associated with a hepatic disease making chickens a convenient homologous small animal model system for the study of HEV pathogenesis and replication. The clinical and pathological findings associated with avian HEV infection in chickens under the natural route of infection have been reported (Billam et al., 2005). The complete genomic sequence of avian HEV was determined and found to be similar to that of mammalian HEVs (Huang et al., 2004). In spite of an approximately 50% nucleotide sequence identity with mammalian HEVs, avian HEV shares many significant structural and functional features with human and swine HEVs. Avian HEV also shares approximately 80% nucleotide sequence identity with the Australian chicken big liver and spleen disease virus (BLSV) (Haqshenas et al., 2001; Payne et al., 1999).

Recently we found that antibodies to avian HEV were also prevalent in healthy chicken flocks in the United States (Huang et al., 2002b), suggesting that chickens in the United States are subclinically infected. From a recent prospective study, we isolated an
apparently avirulent strain of avian HEV from healthy chickens without clinical disease (Sun et al., 2004). Chickens became infected with this apparently avirulent virus at about 3-4 months of age under natural conditions. However, the clinical signs of HS syndrome in infected chickens were absent. Since the avian HEV strain from healthy chickens does not cause any clinical disease, it is important to determine its complete genomic sequence and compare it to the pathogenic strain from chicken with HS syndrome. Thus far, the complete genomic sequence for avian HEV is reported only for a single pathogenic strain, the prototype avian HEV (Huang et al., 2004).

**Attributions:** I acknowledge the attributions of the following people. Z. F. Sun for the pooled bile and fecal suspensions used in the study and X.J. Meng for intellectual help.

**MATERIALS AND METHODS**

**Virus.** The original virus material used for the determination of the complete genomic sequence of the apparently avirulent avian HEV was collected from healthy chickens in a prospective study (Sun et al., 2004). The original virus was further biologically amplified by infection of young SPF chickens to produce a larger virus stock. The virus recovered from the healthy chickens from a Virginia farm was inoculated into seven young SPF chickens. Six out of seven chickens had seroconverted by 18 weeks post-inoculation (wpi). The fecal and bile suspension harvested from two of the chickens at 3 wpi was tested positive by RT-PCR for avian HEV RNA, and was subsequently pooled to generate a virus stock for use in this study.
**Primer Design.** Primers used for the amplification and subsequent sequencing of genomic fragments of the avirulent avian HEV strain in this study were designed on the basis of the prototype pathogenic avian HEV (Table 1). Commercially available primers were used for 5' RACE and 3' RACE to determine the 5’-end and 3’-end sequences.

**RT-PCR.** Briefly, RNA was extracted with TriZol Reagent (MRC) from 200 µl of virus stock. Total RNA was resuspended in 12.25 µl of DNase, RNase, and proteinase-free water (Invitrogen). Reverse transcription was performed at 42°C for 60 min with 1 µl of reverse primer, 0.5 µl of superscript II reverse transcriptase (Invitrogen), 1 µl of 0.1M dithiothreitol, 4 µl of 5x RT buffer, 0.5 µl of RNase inhibitor, and 1 µl of 10 mM dNTPs. Five microliters of the resulting cDNA was amplified in a 50 µl reaction using Platinum High Fidelity Supermix (Invitrogen) by employing two-round nested PCR. The details of the primers used in the study are listed in Table 1.

**Amplification of the extreme 3' end of the viral genome by 3' RACE.** To identify the extreme 3’ genomic sequence, we employed the 3’ RACE technique (Rapid amplification of cDNA ends) using a commercially available 3’ adapter and outer and inner antisense primers (Ambion). Total RNA extracted from 200 µl of the avirulent avian HEV material was resuspended in 9 µl of DNase, RNase, and proteinase-free water (Invitrogen). RNA was then reverse-transcribed at 55°C using 1 µl thermostable reverse transcriptase, Thermoscript (Invitrogen) in the presence of a 20 µl reaction mixture consisting of 2 µl of 10 µM 3’ adapter, 4 µl 5X cDNA synthesis buffer, 1µl DTT, 1 µl RNaseOUT and 2 µl of dNTPs. Nested PCR was performed using Platinum High Fidelity Supermix.
(Invitrogen). Outer 3' RACE antisense primer and avirulent avian HEV-specific forward primer F6227 were used in first round PCR amplification with the following PCR conditions: initial incubation at 94°C for 9 min, followed by 39 cycles of denaturation at 94°C for 0.5 min, annealing at 46°C for 0.5 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Inner 3' RACE reverse primer and avirulent avian HEV-specific inner forward primer FF6307 were employed in a second-round nested PCR with essentially the same PCR conditions except for an annealing temperature at 48°C.

**Amplification of the extreme 5' end of the viral genome by 5' RACE.** The genomic sequence at the extreme 5' end was determined by using the 5' RACE technique (Invitrogen). Total RNA was resuspended in 9 µl of DNase, RNase, and proteinase-free water (Invitrogen). First strand cDNA synthesis was performed by reverse-transcription at 55°C using 1 µl thermostable reverse transcriptase, Thermoscript (Invitrogen) in the presence of a 20 µl reaction mixture consisting of 2 µl of 10 µM avirulent avian HEV-specific reverse primer R355, 4 µl 5X cDNA synthesis buffer, 1 µl DTT, 1 µl RNaseOUT and 2 µl of dNTPs. The cDNA was then purified using a S. N. A. P column (invitrogen). The 3' end of cDNA was tailed with homopolymeric dCTP using TdT (Terminal deoxynucleotidyl transferase) by incubating at 37°C for 10 min. The cDNA was denatured at 94°C for 3 min before TdT tailing to disrupt potential secondary structure. Nested PCR was performed on dC-tailed cDNA using Platinum High Fidelity Supermix (Invitrogen). First-round PCR was done with avirulent avian HEV-specific reverse primer R339 and abridged anchor primer (AAP) (Invitrogen) with the following PCR conditions: initial incubation at 94°C for 9 min, followed by 10 cycles of denaturation at
94\(^0\)C for 0.5 min, annealing at 50\(^0\)C for 0.5 min, extension at 72\(^0\)C for 1 min, followed by 25 cycles of denaturation at 94\(^0\)C for 0.5 min, annealing at 55\(^0\)C for 0.5 min, extension at 72\(^0\)C for 1 min and a final extension at 72\(^0\)C for 7 min. Second-round nested PCR was done using reverse primer R252 and abridged universal amplification primer (AUAP) (Invitrogen) with the following PCR conditions: initial incubation at 94\(^0\)C for 9 min, followed by 39 cycles of denaturation at 94\(^0\)C for 0.5 min, annealing at 58\(^0\)C for 0.5 min, extension at 72\(^0\)C for 1 min, and a final extension at 72\(^0\)C for 7 min.

**DNA sequencing.** The PCR products were excised from 0.8% agarose gel, purified using GeneClean III kit (Qbiogene) and sequenced at the Virginia Bioinformatics Institute Core Laboratory Facility using an automated DNA sequencer (Applied Biosystems). The primer walking strategy was employed to determine the complete genomic sequence of both DNA strands.

**Sequence and phylogenetic analyses.** The complete genomic sequence was assembled and analyzed using DNASTAR and MacVector computer programs (Oxford Molecular Inc.). The consensus sequence was obtained by assembling approximately 19 overlapping sequences, and each sequence, in majority of cases, is based on at least four sequence reads, 2 each from forward and reverse primers. Multiple nucleotide and amino acid alignments were analyzed using CLUSTAL W of the MacVector program.

Phylogenetic analyses were performed using PAUP program (David L. Swofford, Smithsonian Institute, Washington, DC). Phylogenetic tree was constructed using the branch-and-bound search and mid-point rooting options.
GenBank accession numbers. The Genbank accession numbers of the HEV strains used in the sequence and phylogenetic analyses are: Prototype avian HEV (AY535004), Avian hepatitis E virus isolates with partial sequence available: Guelph 01 helicase gene (AY671802), Guelph02 helicase gene (AY671803), Guelph01 capsid gene (AY671804), Guelph02 capsid gene (AY671805), WI318B capsid protein gene (AY870831), WI966B capsid protein gene (AY870829), WI966G capsid protein gene (AY870830), CA889 capsid protein gene (AY870826), CA242 capsid protein gene (AY870819), CA708A capsid protein gene (AY870825), CA077 capsid protein gene (AY870818), CA697A capsid protein gene (AY870822), CA697B capsid protein gene (AY870823), CA697C capsid protein gene (AY870824), NY449 capsid protein gene (AY870828), Arkell swine HEV (AY115488), JKN-Sap (AB074918), prototype swine HEV (AF082843), US1 (AF060668), US2 (AF060669), Hyderabad (AF076239), Burma (M73218), Sar-55 (AF444003), T1 (AJ272108), Morocco (AY230202), Mexico (M74506), JAK-Sai (AB074915), JKK-Sap (AB074917) and swJ13-1 (AB097811).

RESULTS

Determination of the complete genomic sequence of an apparently avirulent strain of avian HEV isolated from healthy chickens

Sequence analysis of the partial helicase gene region of the avian HEV isolates recovered from fecal and serum samples of a healthy chicken revealed a nucleotide sequence identity of only 86% with the prototype pathogenic avian HEV (Sun et al., 2004). A primer walking strategy using primers designed on the sequence of prototype pathogenic avian HEV was employed to determine the complete genomic sequence of the
avirulent avian HEV isolate from a healthy chicken. The extreme 5' and 3' sequences were successfully determined by RACE method. A thermostable reverse transcriptase, Thermoscript RT, was used in the reverse transcription step to melt down the secondary structures and to generate authentic cDNA fragments. Sequences of 63 overlapping fragments were generated and assembled to obtain a consensus sequence.

The complete genome of the avirulent strain of avian HEV, excluding the 3' poly(A) tail, is 6,649 nt in length, 5 nts shorter than the prototype pathogenic strain of avian HEV and 605 nt shorter than human and swine HEVs. Similar to the prototype pathogenic avian HEV and mammalian HEVs, the genome of the avirulent avian HEV consists of a short 5' non-coding region (NCR), followed by three ORFs and a 3' NCR. The 5' NCR is 24 nt and is same in length to that of pathogenic avian HEV. The ORF1 began at nt 25 and ended at nt 4617 and encoded a polyprotein of 1530 aa in length, one amino acid shorter than that of ORF1 of the prototype pathogenic avian HEV. ORF2, from nt 4704 to 6524, was 1821 nt in length, and encoded a putative capsid protein of 606 aa, identical in length to prototype avian HEV. ORF3, from nt 4651 to 4914, comprised of 264 nt and like the prototype avian HEV, encoded a small 87 aa long polyprotein. The ORF2 overlaps with ORF3 as in other HEVs. The 3' NCR, excluding the poly A tail, was 128 nt in length, two nucleotides shorter than the pathogenic strain of avian HEV. The nucleotide sequence identities of the avirulent strain of avian HEV with mammalian HEVs and prototype pathogenic avian HEV were presented in Table 2.

Sequence analyses identified major genetic differences between the prototype pathogenic strain of avian HEV and the avirulent strain
**ORF1:** Compared to the prototype pathogenic avian HEV, a total of 41 mutations were observed in the ORF 1 region (Fig. 1), with nucleotide sequence identity ranging from 89 to 92% in different functional domains such as methyl transferase, helicase and RdRp (Table 3). Two non-silent mutations, V187I and S201T, were identified in the methyl transferase domain. Compared to the prototype avian HEV, papain-like cysteine protease domain of the avirulent strain had a single mutation, K498R, while helicase gene region underwent 3 mutations, V854I, I936M, R950K. RdRp domain encoded 483 a.a and had 6 non-silent mutations: L1071S, V1106A, I1171V, G1179E, D1382E and R1402H. Multiple deletions and insertions were also noted in the avirulent strain. The hypervariable region (HVR) of the avirulent avian HEV was positioned 8 aa ahead of that in the prototype pathogenic avian HEV. A 3-nucleotide deletion coding for glycine in the prototype avian HEV was also observed in the HVR of the avirulent strain, but it had no effect on the reading frame. The HVR region contains 23 non-silent mutations.

**ORFs 2 and 3:** The ORF2 capsid gene is identical in length to the prototype avian HEV, and shares a nucleotide sequence identity of 91%. A total of six non-silent mutations were observed in the ORF2 capsid gene between the prototype strain and the avirulent strain (Table 4), and one of them (C4R) was in the predicted signal peptide region. A R600K mutation occurred in the antigenic domain IV (Haqshenas et al., 2001). The tetrapeptide APLT is conserved, as is the case in other strains of HEV. A 54 aa deletion present in the prototype avian HEV compared to mammalian HEVs was also observed in the avirulent strain.
ORF 3, the smallest of the three ORFs, shared a nucleotide sequence identity of 97% with the prototype pathogenic avian HEV. A total of 4 non-silent mutations were identified between the prototype strain and the avirulent strain (Table 4).

**5' and 3' NCRs:** The 5' NCR sequence of the avirulent strain is identical in length to that of the prototype avian HEV but the 3' NCR has 2 nt deletions at positions corresponding to nt 6558 and 6612, respectively, of the prototype avian HEV.

**Phylogenetic analyses**

Phylogenetic analysis revealed that the avirulent avian HEV clustered together with the prototype avian HEV but was distantly related to mammalian HEVs (Fig. 2a). The avian HEV isolates from different geographic locations, the prototype avian HEV and the avirulent avian HEV isolated from a healthy chicken are heterogeneous in the capsid gene (Fig. 2b).

**DISCUSSION**

Avian HEV was originally identified from chickens with HS syndrome in the United States (Haqshenas *et al.*, 2001). HS syndrome, also called as necrotic, hemorrhagic, hepatomegalic hepatitis, causes mortality in broiler breeder hens and laying hens of 30 to 72 weeks of age and is characterized by gross lesions like regressive ovaries, enlarged liver and spleen, and blood in the abdomen (Riddell, 1997). The discovery of avian HEV associated with a hepatic disease provided a homologous small animal model system to study HEV pathogenesis and replication (Billam *et al.*, 2005). Unfortunately, only a single strain of avian HEV has thus far been fully sequenced.
(Huang et al., 2004). The prototype avian HEV shares significant structural and functional motifs with mammalian HEVs (Haqshenas et al., 2001; Huang et al., 2004), indicating that it belongs to the same genus as human and swine HEVs, *hepevirus* (Emerson et al., 2004). A previous seroepidemiological study revealed that avian HEV is enzootic in chickens in the United States (Huang et al., 2002b). The identification of avirulent avian HEV isolates from a clinically healthy chicken farm (Sun et al., 2004) prompted us to determine the complete genomic sequence of this avirulent strain of avian HEV for better understanding the genetic basis of attenuation.

Sequence analyses revealed that there exist major genetic changes between the avirulent strain and the prototype pathogenic avian HEV. The complete avirulent avian HEV genome was 605 bp shorter than mammalian HEV strains and 5 nts shorter than the prototype avian HEV. Like the prototype avian HEV, the avirulent avian HEV shares significant features with mammalian HEV strains. The 5' NCR region of the avirulent strain is identical in size and sequence to the prototype avian HEV but very different from the highly conserved 5' NCRs of mammalian HEV strains. Apart from the deletions and mutations observed in the ORF1 of prototype avian HEV, an additional 3 nt deletion for an amino acid glycine with no change in the reading frame, were also noted in the avirulent avian HEV. The HVR region was the most divergent region not only with the mammalian HEVs, but also with the pathogenic avian HEV. The role of the HVR remains unknown. The 3' NCR region of the avirulent strain has an additional 2 nt deletion.

The ORF1 gene of the avirulent avian HEV contains the majority of mutations compared to the prototype avian HEV. A total of 41 mutations occurred in ORF1, with
nine of them occurred in helicase and RdRp genes, the regions responsible for virus replication. The identification of specific non-silent mutations that are responsible for virus virulence and pathogenesis of many viruses enabled extensive studies aimed at the attenuation of highly pathogenic viruses. A single or a few amino acid changes were found to alter the virulence and attenuation phenotypes, tissue tropism, cell culture adaptation, \textit{in vitro} growth characteristics and temperature sensitivity. In hepatitis B virus (HBV), the amino acid at position 306 in reverse transcriptase gene was found to be responsible for regulation of replicative competency of HBV isolates (Lin \textit{et al.}, 2005). A number of mutations in the HBV polymerase gene have been determined to affect viral replication (Sheldon \textit{et al.}, 2006). Similarly, the Y73H mutation in 3D\textsuperscript{pol} region of a temperature-resistant mutant S138C\textsubscript{5} of Sabin type 1 polio virus strain was found to contribute to the neurovirulence phenotype in monkeys. This mutation was also found in the neurovirulent wild-type Mahoney strain, but not in the attenuated phenotype Sabin type 1 (Christodoulou \textit{et al.}, 1990). Point mutations leading to G108V or G108D amino acid changes in putative N-terminal cleavage site of the porcine transmissible gastroenteritis virus (TGEV) replicase resulted in a three logarithm reduction in virus titer (Galán \textit{et al.}, 2005). The identified critical mutations in the functional domains of ORF1 encoding proteins and enzymes involved in virus replication of the avirulent avian HEV may influence and modulate the replicative competency and may be responsible for the existence of the naturally-occurring attenuation phenotype of avian HEV. Clearly, additional studies are warranted to systematically determine which mutation(s) in the ORF1 are critical for virus attenuation.
Six unique non-silent mutations were identified in the capsid region (ORF2) of the avirulent strain. One of the non-silent mutations (R600K) is located in the putative antigenic domain IV of the prototype avian HEV and another in the putative signal peptide (C4R) that is necessary for translocation of the peptide into the endoplasmic reticulum. Cysteine residues form structural domains important for protein functions by covalent disulphide bonds. The mutation from a hydrophobic cysteine to highly charged hydrophilic arginine (C4R) could potentially alter the signal peptide secondary structure and function. The R600K mutation in the antigenic domain IV might have effect on the virulence characteristics of this isolate, and thus making it attenuated. Virus capsid is the major determinant of the virus virulence and it is well known that mutations in the capsid gene can attenuate many viruses. It has been shown that the genetic basis of the attenuation phenotype of Polio virus, serotype 3, lies in a S91F amino acid substitution in the coat protein VP3 along with a point mutation in the noncoding region (Westrop et al., 1989). Another Sabin 3-specific amino acid mutation I6T of capsid protein VPI was also found to be contributing to the attenuating phenotype of this vaccine strain (Tatem et al., 1992). It is interesting to note that just a few amino acid mutations drastically diminished the neurovirulence of this strain and make it safe enough to use as a vaccine. In foot-and-mouth disease virus, the amino acid arginine at codon 56 in the capsid protein VP3 was reported to be critical for cell tropism and plaque morphology, making the variant bearing the arginine residue attenuated in vivo in cattle (Sa-Carvalho et al., 1997). The capsid gene mutations P110A and R191S in porcine circovirus type 2, a DNA virus, attenuated the virus in vivo when experimentally inoculated into specific pathogen free pigs (Fenaux et al., 2004). There are ample examples in the literature where a single or a few amino
acid changes on the capsid gene can alter virus virulent phenotypes (Almond et al., 1987; Ceres et al., 2004; Ferreira et al., 2003; Macadam et al., 1991; Minor et al., 1989; Suk et al., 2002; Tzeng & Frey, 2005; van Loon et al., 2002; Wada et al., 1994; Yamaguchi et al., 2001). The identification of 6 unique non-silent mutations in the capsid gene of the avirulent avian HEV suggests that they may play a potential role in determining the attenuation phenotype of this avirulent avian HEV. Further experiments, beyond the scope of this study, to characterize these 6 non-silent mutations on the capsid gene will be helpful in understanding the genetic basis of HEV attenuation.

In summary, we determined the complete genomic sequence of the avirulent strain of avian HEV and identified critical amino acid changes that may be important in HEV virulence. This is only the second strain of avian HEV that has been fully sequenced so far. The discovery of pathogenic avian HEV from chickens with hepatic disease (HS syndrome), the identification and characterization of an avirulent strain of avian HEV from apparently healthy chicken, and the availability of the complete genomic sequence of this avirulent strain of avian HEV will now afford us the opportunity to definitively identify the genetic determinants of HEV virulence, and aid HEV vaccine development in the future.

ACKNOWLEDGEMENTS

This study was supported by grants to X.J.M. from the National Institutes of Health (AI050611 and AI065546).
REFERENCES


**Fig. 1.** Amino acid sequence alignment of ORF1 of the prototype avian HEV and the avirulent avian HEV. Identical amino acids are indicated by grey shadow and the mutations are shown by asterisks. Deletions are denoted by dashes. The first and the last nucleotide positions of putative functional domains and HVR region are indicated by arrows. HVR is underlined. The putative functional domains are: Methyl = Methyl transferase; PLP = Papain-like cysteine protease; HVR = Hypervariable region; Helicase = Helicase; RdRp = RNA-dependent RNA polymerase.
Fig. 2. (a) A phylogenetic tree based on the complete genomic sequences of the avirulent avian HEV, the prototype avian HEV and selected mammalian HEV strains. (b) A phylogenetic tree based on the partial capsid gene sequences of the avirulent avian HEV, prototype avian HEV and other avian HEV isolates from the United States and Canada. The trees were constructed with the aid of PAUP program using branch-and-bound search with mid-point rooting option. A scale bar representing the number of character state changes is proportional to the genetic distance. The HEV genotypes are indicated in the parantheses.
Table 1. Oligonucleotide primers used for cDNA amplification, nested PCR and DNA sequencing of avirulent avian HEV

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<th>Primer</th>
<th>Sequence(5′ → 3′)</th>
<th>Position(nt)*</th>
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<td>5′ RACE outer primer(AAP)</td>
<td>GGGCCACCGTCGACTAGTACCGGGIIGGGGIIGGGIIGG</td>
<td>Non-viral primer</td>
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*The position is relative to the prototype pathogenic strain of avian HEV

(Huang et al., 2004).
### Table 2. Pairwise sequence comparison of the full-length genomic sequence of avirulent avian HEV with other selected representative HEV strains. The values indicate the nucleotide sequence identities

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<th>Mexico</th>
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<th>US2</th>
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<th>T1</th>
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* % nucleotide sequence identity
Table 3. Nucleotide sequence identities of avirulent avian HEV with the prototype avian HEV in different regions of the genome

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Table 4. Amino acid mutations in ORF2 and ORF3 genes of the avirulent strain of avian HEV compared to the prototype pathogenic avian HEV

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Chapter 5

Comparative pathogenesis in specific-pathogen-free chickens of avian hepatitis E virus isolates recovered from a chicken with Hepatitis-Splenomegaly syndrome and from a healthy chicken

P. Billam, S. Pudupakam, T. LeRoith, F.W. Pierson, R. B. Duncan and X.J. Meng

To be submitted to Journal of Virology

ABSTRACT

Recently, an apparently avirulent strain of avian HEV was identified from healthy chickens and genetically characterized. In this study, an infectious stock of avirulent avian HEV was generated and infectivity titration determined in young specific-pathogen-free (SPF) chickens. To compare the pathogenicity of the pathogenic strain and the apparently avirulent strain, six-week-old SPF chickens were inoculated with one of two strains of avian hepatitis E viruses, pathogenic avian HEV recovered from a chicken with HS syndrome and avirulent avian HEV isolated from a healthy chicken. Fifty four chickens were assigned to three groups. Group 1 chickens \((n = 18)\) were each intravenously inoculated with \(5 \times 10^{2.5}\) 50% chicken infectious dose of pathogenic avian HEV, group 2 chickens \((n = 18)\) were each inoculated with the same dose of avirulent avian HEV and group 3 chickens \((n = 18)\) were not inoculated and were used as controls. Six chickens from each group were necropsied at 2, 3 and 4 weeks post-inoculation (wpi). Apart from weekly sera and fecal swabs, various tissues including liver and spleen were collected at necropsy for histopathological evaluation. Most of the chickens seroconverted by 3 wpi in both pathogenic avian HEV and avirulent avian HEV groups.
The mean antibody titers for both inoculated groups peaked at 3 wpi and were higher for pathogenic avian HEV compared to the avirulent avian HEV group. Avian HEV RNA was detected in feces and serum of the chickens from both the inoculated group from 1 wpi. Liver histopathological lesions were mainly lymphocytic periphlebitis and phlebitis. The liver and spleen lesion scores were significantly different between the treatment groups ($P = 0.03$ and $P = 0.0006$ respectively)) and the overall hepatic lesion score was higher for the pathogenic avian HEV group compared to the avirulent avian HEV and control groups. Creatine phosphokinase (CPK) levels were significantly different between the groups ($P = 0.049$) and the mean CPK values were higher for the pathogenic avian HEV group than the avirulent avian HEV and control group. Based on the evaluation of liver microscopic lesions and profiles of antibody levels, the pathogenic avian HEV induced relatively severe hepatic lesions and elicited higher level of humoral immune response than the avirulent avian HEV, and thus suggestive of attenuation.
INTRODUCTION

Hepatitis E is an acute, enterically transmitted disease characterized by jaundice, anorexia, malaise, fever, nausea and abdominal tenderness in humans (13, 22, 25). Hepatitis E virus (HEV), the causative agent of hepatitis E, is a positive-sense, non-enveloped RNA virus. The genome size is 7.2 kb and has three open reading frames (ORFs) (13, 36, 37). Hepatitis E is epidemic and endemic in many developing countries due to poor sanitation and contaminated water supply. Major hepatitis epidemics occurred in the developing countries of Asia, Africa and North America (5, 13, 36) and sporadic cases of acute hepatitis E have also been reported in industrialized countries including the United States (18, 30-32, 36, 41, 42). HEV mainly affects young adults, causing a 15-25% (1, 23, 26) and 1% mortality (13) in infected pregnant women and in general population, respectively. The primary mode of transmission is by fecal-oral route (3, 5, 13, 19, 36), though blood-borne (24, 28) and food-borne (29, 47-49, 55) transmissions have also been documented.

Meng et al. (33) discovered the first animal strain of HEV, the swine HEV from commercial swine in USA, and the swine HEV sequence was significantly different from HEV isolates from Asia and Mexico. Since then, many strains of HEV have been isolated from pigs in different geographical regions of the world along with human strains, and have been shown to be closely related to human strains of HEV (4, 8, 10, 11, 14, 31, 33, 34, 45, 46, 51, 52, 54, 56-58). Another animal strain of HEV, avian HEV, was isolated and characterized from chickens with Hepatitis-Splenomegaly (HS) syndrome (17). HS syndrome is an emerging disease of commercial egg laying hens of 30-72 weeks of age in North America and is characterized by ovarian regression, enlarged liver and spleen and
red fluid in the abdomen (38-40). The complete sequence of avian HEV was determined and was reported to be similar to that of mammalian HEVs with approximately 50% nucleotide sequence identity (21). Apart from functional and structural similarities to human and swine HEVs, avian HEV also shares common antigenic epitopes in the capsid protein (15, 16). Avian HEV also shares approximately 80% nucleotide sequence identity with the Australian chicken big liver and spleen disease virus (BLSV) (17, 35). Sequence analysis of a strain of avian HEV recovered from an outbreak of HS syndrome revealed sequence identities of 82-92% and 78-80% in helicase and capsid genes respectively compared to other avian HEV isolates (2). Avian HEV belongs to the genus *Hepevirus* (12), and phylogenetic analyses of avian HEV isolates identified from chickens in United States indicate that they belong to a putative new genotype 5 or a separate species (20). The discovery of avian HEV associated with a hepatic disease provided a homologous small animal model system to study HEV replication and pathogenesis under the natural route of infection (6).

Recently, prevalence of antibodies to avian HEV was also reported in healthy chicken flocks in the United States (20), suggesting that chickens in the United States are subclinically infected with HEV or an HEV-like agent. From a recent prospective study, an apparently avirulent strain of avian HEV was isolated from healthy chickens without clinical disease (43). The complete genomic sequence of the apparently avirulent avian HEV was reported and was found to have a sequence identity of 90.1% with the prototype pathogenic strain (7). The objective of this study is to compare the pathogenicity between the apparently avirulent strain and the prototype pathogenic strain of avian HEV in specific-pathogen-free chickens under experimental conditions.
Attributions: I acknowledge the attributions of the following people. S. Pudupakam for help with chicken experiment T. LeRoith for histopathological evaluation, F.W. Pierson, R.B. Duncan and X.J. Meng for intellectual help.

MATERIALS AND METHODS

Virus stock of the prototype pathogenic avian HEV. The pathogenic avian HEV used in this study was originally recovered from a bile sample obtained from a 56-week-old chicken with HS syndrome. An infectious stock of avian HEV was generated and titrated in young specific-pathogen-free (SPF) chickens (44). The prototype avian HEV stock with an infectious titer of $5 \times 10^{2.5}$ 50% chicken infectious dose (CID$_{50}$) per ml was used as inocula for this study.

Generation of an infectious stock of an apparently avirulent strain of avian HEV. In order to compare the pathogenicity of the prototype and the apparently avirulent avian HEV strains, an infectious stock of the avirulent strain of avian HEV with known infectious titer had to be produced. The original virus material used for the generation of an infectious stock of the apparently avirulent avian HEV was collected from healthy chickens in a prospective study (43). The virus recovered from the healthy chickens from a Virginia chicken farm was experimentally inoculated into young SPF chickens. The fecal and bile suspension from two of the inoculated chickens which tested positive by RT-PCR were harvested and was subsequently pooled to generate a virus stock. The virus stock was further biologically amplified by inoculation of two additional young SPF chickens. The feces were collected from the two inoculated chickens on alternate days.
and were tested by RT-PCR for avian HEV RNA. The chickens were necropsied when
fecal virus shedding was detected by RT-PCR, and during necropsies bile and intestinal
contents were collected and used to make a 10% suspension in 10% PBS buffer (w/v) as
the virus stock. The infectious stock was subsequently titrated in SPF chickens to
determine its infectivity titer.

**Infectivity titration of the apparently avirulent strain of avian HEV in SPF
chickens.** Since HEV cannot be propagated *in vitro*, a chicken bioassay was used to
determine the infectivity titer of the avirulent avian HEV stock. Briefly, twenty-four
young SPF chickens (Charles River SPAFAS Inc., Wilmington, MA) were divided into 6
isolators containing 4 birds each. The avirulent avian HEV infectious stock was serially
diluted 10 fold from $10^{-1}$ to $10^{-5}$ in PBS buffer. Each of four chickens in an isolator was
intravenously inoculated with 200 µl of each dilution of infectious stock and the last set
of 4 control chickens were inoculated with 200 µl of PBS buffer. Weekly blood and fecal
swabs were collected from each chicken. ELISA was performed on the weekly sera to
detect anti-avian HEV antibodies, and RT-PCR was done on the weekly sera and fecal
materials to detect avian HEV RNA. The inoculated chickens were monitored for 10
weeks for evidence of virus infection. The infectivity titer of avirulent avian HEV stock
was calculated as 50% chicken infectious dose ($\text{CID}_{50}$) per ml of the virus inoculum.

**Experimental design for the comparative pathogenesis study.** Fifty four, six-week-
old, SPF chickens were divided into 3 groups of 18 chickens each. Chickens in group 1
(n=18) were each intravenously (IV) inoculated with $5 \times 10^{2.5} \text{CID}_{50}$ of the prototype
pathogenic avian HEV stock. Chickens in group 2 (n=18) were each IV inoculated with $5 \times 10^{2.5}$ CID$_{50}$ of the apparently avirulent avian HEV stock. Eighteen chickens in group 3 served as uninoculated controls. Each group was housed in a separate isolation room and the chickens were allowed access to feed and drinking water *ad libitum*.

**Sample collection and processing.** Blood and fecal swabs were collected from each chicken prior to inoculation and weekly thereafter. Weekly blood plasmas were tested for liver enzyme profiles as previously described (6). Weekly serum samples were tested by ELISA for anti-avian HEV antibodies. Weekly serum samples and fecal swab materials were tested for avian HEV RNA by RT-PCR. Six chickens from each group were necropsied at 2, 3 and 4 wpi, respectively. Tissue samples including liver, thymus, spleen, heart, pancreas, duodenum, jejunum, ileum, cecum and colorectum were collected from each chicken during each necropsy and fixed in formalin for histopathological evaluation.

**Gross pathology and histopathology evaluations.** Livers were evaluated for gross pathological lesions during necropsies as previously described (6) and also recorded as digital pictures. Tissue samples collected at each necropsy were fixed in 10% neutral buffered formalin and processed for routine histopathological examinations. Histopathological lesions in various tissues were evaluated in a blinded fashion by a board-certified veterinary pathologist (T.L.) and were scored according to lesion severity based on standard scoring systems reported previously (6). Liver lesion scores range from 0 to 4 (0, no lesions; 1, < 5 foci; 2, 5-8 foci; 3, 9-15 foci; 4, >15 foci. The no. of foci were
bond on a single shade.). Thymus lesions were given scores from 0 to 4 (0, no foci; 1, 1 to 5 foci; 2, 5-10 foci; 3, 10-20 foci; 4, >20 foci) and spleens were scored from 0 to 3 (0 = normal, 1 = minimal, 2 = moderate, 3 = severe).

**Biochemical profiles.** A total of 18 chickens (6 each from pathogenic avian HEV group, avirulent avian HEV group, and control group) were monitored weekly for biochemical evidence of hepatitis from 2 wpi to the end of the study at 4wpi. Plasma levels of liver enzymes for the 18 chickens, including aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine phosphokinase, bile acids, and total proteins were determined by standard methods (Avian and Exotic Animal Clinical Pathology labs, Wilmington, OH).

**ELISA for anti-HEV antibodies.** A purified truncated recombinant ORF2 capsid protein of avian HEV expressed in *Escherichia coli* was used as the antigen in the ELISA to detect avian HEV antibodies in chickens as previously described (16, 20, 43). Briefly, the purified avian HEV antigen was coated onto 96-well plates (Thermo Labsystems, Franklin, MA). Primary antibody was represented by the collected serum samples. A horseradish peroxidase-conjugated rabbit anti-chicken IgG (Sigma Chemical Co., St. Louis, Mo.) was used as the secondary antibody. The optical density values were measured at 405 nm. Samples with OD values greater than 0.30 were considered positive (20, 43). Convalescent sera from experimentally infected chickens (6) and sera from SPF chickens were included as positive and negative controls, respectively.
**RT-PCR for detection of avian HEV RNA.** To detect avian HEV RNA in feces and serum, RT-PCR was performed as previously described (20). Briefly, RNA was extracted with TRI reagent (MRC) from 100 µl of serum or 10% fecal suspension. Total RNA was resuspended in 12.25 µl of DNase, RNase, and proteinase-free water (Invitrogen). Reverse transcription was performed at 42°C for 60 min with 1µl (10 pm) of reverse primer (pathogenic avian HEV specific P2 (5’ -ACAGTTTCACCTCAGGCTCG -3’)) or avirulent avian HEV specific YR (5’ -CTGCACACGTATCCATTAG -3’), 0.25 µl [50 U] of superscript II reverse transcriptase (Invitrogen), 1 µl of 0.1M dithiothreitol, 4 µl of 5x RT buffer, 0.5 µl [20 U] of RNasin RNase inhibitor (Promega), and 1 µl of 10 mM dNTPs. Five microliters of the resulting cDNA was amplified in a 50 µl reaction using Platinum High Fidelity Supermix (Invitrogen). For detection of viral RNA in chickens inoculated with pathogenic avian HEV, the first round PCR produced an expected fragment of 595 bp with the forward primer P1 (5’-ACAACATCCACCCCTACAAG-3’) and the reverse primer P2. For the second round PCR, the forward primer P3 (5’-AGAACAATGGTTGGCAGGCTCC-3’) and the reverse primer P4 (5’-GAGGGCAAGCCACCTAAAAC-3’) amplified an expected fragment of 394 bp. The PCR parameters included an initial incubation at 94°C for 9 min, followed by 39 cycles of denaturation at 94°C for 0.5 min, annealing at 52°C (56°C for 2nd rd PCR), 0.5 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min.

For detection of viral RNA in chickens inoculated with avirulent avian HEV, forward primer YF (5’- GCTGCCCTTGGGATGTTCAT-3’) and the reverse primer YR were used in the first round PCR to produce an expected fragment of 712 bp. For the second round PCR, the forward primer YF2 (5’-AGTTTTGCCTCTGCTGTTT-3’).
and the reverse primer YR2 (5’-AGCGTGTATTACCCGCAAGGC-3’) amplified an expected fragment of 578 bp. The PCR conditions were essentially the same as described above, except for annealing temperatures of 48°C and 54°C in 1st and 2nd rounds, respectively. The PCR products amplified from serum and feces of selected chickens from each of inoculated groups were sequenced to confirm the identity of the virus recovered from the experimentally infected chickens.

**DNA sequencing.** The PCR products were excised from 0.8% agarose gel, purified using Geneclen III kit (Qbiogene), and both strands were sequenced at the Virginia Bioinformatics Institute Core Laboratory Facility using an automated DNA sequencer (Applied Biosystems).

**Statistical analyses.** Histopathologic lesions were recorded as lesion scores. The means of lesion scores were compared by weighted least squares analysis of variance using the CATMOD procedure of SAS® (version 9.1.3; SAS Institute, Inc., Cary, N.C.). Antibody titers were compared by repeated measures analysis of covariance using baseline titers as co-variates by the GLIMMIX procedure. A box-cox transformation was applied to antibody titers to obtain approximate gaussian distribution. The biochemical profiles of liver enzymes, total proteins and bile acids were compared by repeated measures analysis of variance using the GLIMMIX procedure. Models included group (GRP) and WPI and GRP x WPI interaction.

**RESULTS**
**Generation of an infectious stock of avirulent strain of avian HEV.** Due to the low titer of the virus in the original stock from a prospective study, the original pooled feces and bile suspension was experimentally inoculated into two young SPF chickens to generate an infectious stock. Avian HEV RNA was detected in feces from 3 dpi. The RT-PCR positive bile and fecal suspension obtained from necropsy of one chicken at 23dpi and the second chicken at 30 dpi was pooled to make a 10% suspension in PBS buffer as an infectious stock.

**Infectivity titration of an infectious stock of avirulent avian HEV in young SPF chickens.** All the chickens inoculated with $10^{-1}$ and $10^{-2}$ dilutions of the avirulent avian HEV infectious stock seroconverted (Table 1). However, the chickens in the other groups including the control chicken remained seronegative throughout the study. One of four chickens inoculated with the largest dose ($10^{-1}$ dilution) seroconverted at 5 wpi, and all of the remaining chickens seroconverted by 6 wpi. In the group inoculated with $10^{-2}$ dilution of the infectious stock, one chicken was seropositive from 1 wpi, 2 chickens were seropositive by 4 wpi (one of the two showed baseline titers by 7 wpi). The other two chickens started seroconverting at 6 and 7 wpi respectively. Viremia and fecal shedding of the virus was observed in the chickens inoculated with the highest dilutions, but not in the group inoculated with $10^{-3}$ dilution (Table 2). The chickens inoculated with $10^{-4}$ and $10^{-5}$ dilution and PBS (control) were not tested as they remained seronegative the entire duration of the study. Seroconversion in the inoculated chickens was used as the end point for the calculation of the infectivity titer of the infectious stock. The infectious titer for the avirulent strain of avian HEV was determined to be $5 \times 10^{2.5} \text{CID}_{50}$ per ml.
Comparative pathogenesis study:

**Gross and microscopic lesions.** Gross lesions were observed in only two inoculated chickens at 2 wpi. One chicken from the pathogenic avian HEV group had a localized circular blotched area on left liver lobe, and subcapsular hemorrhages were found in the right lobe of liver of chicken inoculated with avirulent avian HEV. Both of the chickens had high scores for histopathological lesions (4 and 3 respectively).

The microscopic lesions in liver included lymphocytic phlebitis, lymphocytic and heterophilic periphlebitis and fibrinoid necrosis (Fig. 1A and 1B) and were relatively mild in the avirulent avian HEV group. Subcapsular hemorrhages, massive necrosis or amyloid-like lakes were not observed. The mean lesion scores were higher for pathogenic avian HEV group and avirulent avian HEV group than the control group at 2 and 3 wpi, but not at 4 wpi (Table 3). The lesion scores for pathogenic avian HEV group were higher than the scores for avirulent avian HEV group at 3 and 4 wpi. Overall, the hepatic lesion scores differed between treatment groups ($P = 0.03$) and were higher for the pathogenic avian HEV group than the avirulent avian HEV group (least square mean [LSM] for pathogenic avian HEV group, 2.722; LSM for avirulent avian HEV group, 2.61; LSM for control group, 1.877). However, the lesion scores did not differ over time ($P = 0.48$).

Microscopic lesions were also observed in spleen and thymus. Foci of lymphoid hyperplasia were observed in spleen (Fig. 2). The spleen lesion scores differed between treatment groups ($P = 0.0006$), but did not behave differently over time ($P = 0.32$). Mild cortical hyperplastic lesions were seen in thymus, but the lesion scores did not differ
between treatment groups ($P = 0.27$). Microscopic lesions like periphlebitis and lymphoid hyperplasia were observed in jejunum, cecum and colorectum.

**Seroconversion to anti-HEV antibodies.** The chickens were seronegative for HEV prior to inoculation. Most of the inoculated chickens seroconverted to anti-avian HEV antibodies by 3 wpi (Table 4 and Table 5). By 3 wpi, anti-avian HEV antibodies were detected in 83% (10 out of 12) chickens in pathogenic avian HEV group and 75% (9 out of 12) chickens in avirulent avian HEV group. By 4 wpi, 100% (6 out of 6) chickens in both inoculated groups had seroconverted. OD values differed between the treatment groups ($P =0.0001$) and behaved differently over time ($P = 0.0001$) (for GRP X WPI, $P = 0.0001$). The GRP X WPI effect was significant for both avirulent avian HEV group ($P < 0.0001$) and pathogenic avian HEV group ($P < 0.0001$), but not for the control group ($P =0.11$). The mean OD values for both inoculated groups peaked at 3 wpi (Fig. 3), but was higher for pathogenic avian HEV group (mean OD ± standard error of mean [SEM], 0.847 ± 0.081) compared to the avirulent avian HEV group (mean OD ± SEM, 0.721 ± 0.1).

**RT-PCR detection of avian HEV RNA.** Weekly sera and fecal swabs were tested by RT-PCR using nested set of primers for the presence of avian HEV RNA. Avian HEV RNA was detected in feces and serum of chickens belonging to both the inoculated groups (Table 4 and 5). Viremia and fecal shedding of viral RNA was observed from 1 wpi. Viral RNA was detected in feces and serum of most of the inoculated chickens at 2 wpi. In the pathogenic avian HEV group, fecal shedding of viral RNA was observed in
15 out of 18 chickens at 1 wpi, 18 out of 18 chickens at 2 wpi, 8 out of 12 chickens at 3 wpi and 1 out of 6 chickens at 4 wpi. In the chickens that were inoculated with avirulent avian HEV, feces were RT-PCR positive in 17 out of 18 chickens at 1 wpi, 17 out of 18 chickens at 2 wpi, 7 out of 12 chickens at 3 wpi and 1 out of 6 chickens at 4 wpi. Viremia was observed in 11 at 1 wpi, 17 at 2 wpi, 5 at 3 wpi and 1 at 4 wpi in the chickens inoculated with pathogenic avian HEV and in 11 at 1 wpi, 15 at 2 wpi, 3 at 3 wpi and 0 at 4 wpi in avirulent avian HEV group (out of 18, 18, 12 and 6 chickens respectively). The control chickens were seronegative for anti-avian HEV antibodies during the entire course of experiment, so they were not tested by RT-PCR. PCR products recovered from sera and fecal swabs of selected chickens from both inoculated groups were sequenced and were confirmed to belong to their respective inoculum.

Biochemical profiles of liver enzymes. Liver enzymes including AST and bile acids and total protein from 18 chickens (6 from each group) that were monitored till the end of the study at 4 wpi did not differ between treatment groups (data not shown). LDH values did not differ between treatment groups (\( P = 0.21 \)), but behaved differently over time (\( P = 0.001 \)). However, the creatine phosphokinase (CPK) responses differed between the treatment groups (\( P = 0.049 \)) and behaved differently over time (\( P = 0.043 \)). The mean CPK levels were higher for the pathogenic avian HEV group (\( P < 0.0001 \)) than the avirulent avian HEV (\( P < 0.0001 \)) and control group (least square mean [LSM] for pathogenic avian HEV group, 1686.15; LSM for avirulent avian HEV group, 1402.66; LSM for control group, 1538.31; SEM, 74.39).
DISCUSSION

Avian HEV, isolated from chickens with HS syndrome, produces characteristic gross and microscopic hepatic lesions (6) and provides an excellent homologous small animal model system to study HEV pathogenesis. The identification of an apparently avirulent strain of avian HEV in healthy chickens without clinical symptoms prompted us to determine the complete genomic sequence of the apparently avirulent avian HEV (7). Compared to the prototype pathogenic avian HEV, the avirulent strain has numerous non-silent amino acid mutations in capsid gene and other genomic regions (7). Therefore, it is important to definitively characterize the pathogenicity of the avirulent strain in laboratory conditions and compare its pathogenicity with the prototype pathogenic strain. In this study an infectious stock of avian HEV was first generated and its infectivity titer determined. The pathogenic and avirulent strains of avian HEV were experimentally inoculated into SPF chickens and the differences in pathogenicity with respect to clinical course and pathological lesions were evaluated and compared.

Virus replicated in SPF chickens inoculated with both pathogenic and avirulent strains of avian HEV since the inoculated chickens seroconverted to anti-avian HEV antibodies, and viral RNA was detected in feces and serum. There was no difference in the time point of occurrence of seroconversion or virus shedding in chickens inoculated with both strains of avian HEV, indicating that the avirulent avian HEV virus replicated as rapidly as the pathogenic avian HEV virus. Gross lesions were not prominent, but microscopic hepatic lesions like periphlebitis and phlebitis, the characteristic lesions of avian HEV infection (6), were observed in the inoculated chickens. Significant higher mean scores of liver lesions for chickens inoculated with pathogenic avian HEV indicated
that the hepatic lesions were more severe in the pathogenic avian HEV group compared to avirulent avian HEV group, and thus suggestive of attenuation of the avirulent strain. Interestingly, the attenuation apparently did not affect the replication capacity of avirulent avian HEV suggesting that the difference in pathogenicity was not dependent on replication capacity.

The absence of pronounced gross lesions like subcapsular hemorrhages in the chickens inoculated with the pathogenic avian HEV may be due to the lower dose of the virus (5×10^{2.5} \text{ CID}_{50}) used in the inoculation. Human HEV infection in primates was reported to be dose-dependent with manifestation of biochemical and virological changes of hepatitis in primates that received higher doses of human HEV and subclinical infection in primates with lower doses (50). Recently it was reported that antibodies to avian HEV were also prevalent in healthy chicken flocks in the United States (20) suggesting that chickens in the United States are subclinically infected. It has been suggested that avian HEV alone may not be responsible for classical macroscopic and microscopic lesions associated with HS syndrome, and may involve co-infection with one or a combination of etiological agents (6). The presence of non-specific foci with lymphocytes in the control chickens is not surprising since chickens lack lymph nodes and the lymphoid tissue is dispersed in the gastrointestinal tract and liver. Also, such non-specific background liver lesions were reported previously in chickens (6).

The chickens from both inoculated groups seroconverted and reached the mean antibody titers peaked at 3 wpi, with the chickens inoculated with pathogenic avian HEV reaching higher mean titers compared to the chickens inoculated with avirulent avian HEV (Fig. 3). In contrast to the gradual waning of the mean titers in the pathogenic avian
HEV group at 4 wpi, antibody titers fell sharply for avirulent avian HEV group suggesting that the pathogenic avian HEV elicits slightly persistent immune response compared to the avirulent avian HEV. Also, in the infectivity titration of the avirulent avian HEV in young SPF chickens, antibody titers of four chickens inoculated with the highest dilutions ($10^{-1}$ and $10^{-2}$) of the infectious stock reached the baseline values a few weeks after seroconversion. Viremia and fecal shedding of virus was observed in highest number of inoculated chickens from both groups at 2 wpi, and precedes the attainment of highest antibody titers at 3 wpi. The appearance of humoral response following the detection of virus in feces and serum is consistent with the pathogenesis of HEV infection (13, 36).

The levels of liver enzymes including AST, CPK, LDH, bile acids and total proteins were analyzed. No significant elevations of AST, LDH, bile acids or total proteins were noticed. However, CPK values were significantly different between groups, being higher for the chickens inoculated with pathogenic avian HEV than the control group. Elevated CPK levels indicate muscle injury, and elevation of transaminases suggests liver damage (27). The importance of the elevated CPK levels in the absence of significant AST levels is not known at this time, and it is speculated to be due to non-specific skeletal or cardiac muscle injury.

The complete genomic sequence of avirulent avian HEV contained 6 non-silent mutations in the capsid gene (7), the region containing immunogenic epitopes. The capsid gene mutations, along with mutations in the functional domains of ORF1 may be responsible for the observed differences in the severity of microscopic lesions of liver and for the higher levels of antibody titers in the pathogenic avian HEV group and for the
existence of the naturally-occurring attenuation phenotype of avirulent avian HEV. Presence of microscopic lesions like periphlebitis in the lower GIT of the inoculated chickens indicates the active inflammation and associated replication of the virus. Extrahepatic sites of replication have been identified in chickens under the natural route of infection. Similarly, extrahepatic sites of replication have also been reported in pigs (9, 53). It is believed that the virus first replicates in the GIT following fecal-oral transmission and then reaches liver. Since HEV is not a cytopathic virus, the hepatic damage is believed to be immune-mediated (13).

In summary, the young SPF chickens were successfully infected with two strains of avian HEV, the pathogenic avian HEV and avirulent avian HEV. Overall, the hepatic lesion scores and the antibody titers were significantly higher for the chickens inoculated with pathogenic avian HEV compared to the chickens inoculated with avirulent avian HEV, suggesting that the avirulent strain appeared to be attenuated in experimentally inoculated chickens.

ACKNOWLEDGEMENTS

This study was supported by grants to X.J.M. from the National Institutes of Health (AI050611 and AI065546). We thank Dr. Stephen Werre for help with the statistical analysis of this study. We acknowledge the help of Dr. F. F. Huang, Dr. K. Key, Dr. S. Ramamoorthy and D. Guenette in chicken experiments. We thank Dr. Thomas Toth and Dr. Jake Tu for their support.
REFERENCES


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Fig. 1. Microscopic lesions of the liver. (A) Section of liver from a chicken inoculated with avirulent avian HEV showing focal, mild lymphocytic and heterophilic portal periphlebitis. (B) Section of liver from a chicken inoculated with pathogenic avian HEV showing severe lymphocytic portal phlebitis and periplebitis. The tissues were stained with hematoxylin and eosin.
Fig. 2. Photomicrograph of the spleen from a chicken inoculated with pathogenic avian HEV. Note the coalescing foci of lymphoid hyperplasia surrounding arterioles. This slide was stained with hematoxylin and eosin.
Fig. 3. Seroconversion to avian HEV antibodies in inoculated SPF chickens. The mean ELISA OD values of all chickens from the pathogenic avian HEV, avirulent avian HEV, and control groups at each week postinoculation (wpi) are plotted.
Table 1. Seroconversion to anti-avian HEV antibodies in young SPF chickens experimentally inoculated with different dilutions of an infectious stock of avirulent avian HEV

<table>
<thead>
<tr>
<th>Virus stock dilution</th>
<th>No. of chickens seropositive/no. tested at the following wpi:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
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<tr>
<td>10 -1</td>
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</tr>
<tr>
<td>10 -5</td>
<td>0/4</td>
</tr>
<tr>
<td>Control</td>
<td>0/4</td>
</tr>
</tbody>
</table>
Table 2. Viremia and shedding of avirulent avian HEV in feces in chickens experimentally inoculated with different dilutions of an infectious stock of avirulent avian HEV

<table>
<thead>
<tr>
<th>Virus stock dilution</th>
<th>No. of chickens positive for viremia (shedding of virus in feces)/no. tested at the indicated wpi:</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
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<tr>
<td>$10^{-1}$</td>
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<td>NT</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT: Not tested

The chickens inoculated with lower dilutions ($10^{-1}$ and $10^{-5}$) and PBS (control chickens) were seronegative throughout the entire duration of the study and were not tested by RT-PCR.
Table 3. Microscopic liver lesions in pathogenic avian HEV, avirulent avian HEV and control group chickens\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Group/wpi</th>
<th>No. of chickens with lesions (mean score) at indicated wpi</th>
</tr>
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<tbody>
<tr>
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<td>2</td>
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<tr>
<td>Pathogenic avian HEV group</td>
<td>6/6(2.66)</td>
</tr>
<tr>
<td>Avirulent avian HEV group</td>
<td>6/6(2.83)</td>
</tr>
<tr>
<td>Control group</td>
<td>4/6(1.0)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Six chickens from each group were necropsied at 2, 3 and 4 wpi.

\textsuperscript{b}Only 5 chicken samples were available.

Microscopic liver lesions included lymphocytic periphlebitis and phlebitis.
Table 4. Detection of viral RNA in feces / serum and avian HEV antibodies in the chickens inoculated with prototype pathogenic avian HEV.

<table>
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*Six chickens from each group were necropsied at 2, 3 and 4 wpi.
<table>
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<tr>
<th>Chicken ID No.</th>
<th>Detection of viral RNA in fecal swabs/viremia (detection of avian HEV antibodies) at the following wpi:</th>
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*Six chickens from each group were necropsied at 2, 3 and 4 wpi.
Chapter 6

General Conclusions

Hepatitis E, caused by hepatitis E virus (HEV), is mainly a disease of young adults and the mortality may reach up to 25% in pregnant women. HEV is transmitted by fecal-oral route and waterborne epidemics are the most explosive form in developing countries of Asia and Africa. Serological and genetic identification of HEV strains from various species of animals strongly suggest the existence of animal reservoirs indicating that hepatitis E is a zoonotic disease. The discovery of swine HEV and avian HEV afforded us the use of pigs and chickens as homologous animal model systems to study the replication and pathogenesis of HEV. In this dissertation, chickens have been used as a small homologous animal model system to study the HEV replication and pathogenesis.

We successfully infected SPF chickens experimentally with avian HEV by fecal-oral route, the natural route of transmission. The inoculated chickens seroconverted to avian HEV antibodies and shed viral RNA in feces and serum. Characteristic gross and microscopic lesions were observed. The results indicated that the chickens are a useful model for studying pathogenesis and replication of HEV. We demonstrated for the first time that chickens can be experimentally infected with HEV by the natural fecal-oral route.

We identified extrahepatic sites of replication of avian HEV under the natural route of infection. Replicative negative-strand viral RNA and positive immunohistochemical signal for avian HEV capsid protein were detected in a variety of gastrointestinal tract (GIT) tissues apart from liver, indicating that the virus replicates in
GIT tissues following fecal-oral transmission and that non-liver sites of viral replication exist for HEV. It also appears that avian HEV replicates earlier in extrahepatic tissues followed by replication in liver after oronasal administration. This research evidence is important to explore an ideal and practical in vitro culture system for HEV.

The full-length genomic sequence of an apparently avirulent strain of avian HEV that was isolated from a healthy chicken was determined and analyzed. Sequence comparisons revealed that the avirulent avian HEV is very similar to the pathogenic avian HEV in terms of genomic organization and sequence with a nucleotide sequence identity of 90.1%. Several critical amino acid mutations were observed in the coding regions of all three ORFs, especially the capsid gene (ORF2), which may be responsible for the attenuation of avirulent avian HEV. Phylogenetic analysis indicated that the avirulent avian HEV clusters with pathogenic avian HEV and forms a distinct branch from the mammalian HEVs.

To compare the pathogenicity of the prototype pathogenic avian HEV and the apparently avirulent avian HEV, an infectious stock of avirulent avian HEV was first generated and the infectivity titer was determined in young SPF chickens to be $5 \times 10^{2.5}$ CID$_{50}$ per ml. The infectious stocks of pathogenic avian HEV and avirulent avian HEV were inoculated into young SPF chickens to study the comparative pathogenesis of the two avian HEV strains, one pathogenic, the other avirulent. The inoculated chickens seroconverted to avian HEV antibodies. Fecal shedding of viral RNA and viremia were observed in both inoculated groups. Characteristic hepatic lesions of avian HEV like periphlebitis and phlebitis were also observed with higher mean liver scores for chickens inoculated with pathogenic avian HEV compared to the avirulent and control groups,
suggestive of attenuation. However, the lesions were less severe in the prototype pathogenic group possibly due to low dose of the virus used in the inoculum.

In summary, chickens are an excellent, homologous, small animal model system to study the HEV pathogenesis and replication. Further studies to determine the role of non-silent mutations in the attenuation of avirulent strain are warranted for complete understanding of the genetic determinants of virulence.
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Awards and Honors

1. Best Virology Graduate Student Presentation Award sponsored by the American College of Veterinary Microbiology. 84th Annual Meeting of the Conference of Research Workers in Animal Diseases (CRWAD) in Chicago, IL, Nov 9-12, 2003.

2. First prize for best graduate student oral presentation at 56th Annual Meeting of the Animal Disease Research Workers in the Southern States at Virginia Tech, Blacksburg, VA. April 6-8, 2005.

3. Student Travel Award to attend the 2005 American Society for Virology 24th annual meeting at University Park, PA, June 18-22, 2005.


5. Lions International Gold Medal for highest GPA in the dept. of Medicine in DVM (B. V. Sc)

6. Lady Veterinarian Prize in DVM (B. V. Sc).

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