Antigenic epitope composition and protectivity of avian hepatitis E virus (avian HEV) ORF2 protein and vertical transmission of avian HEV

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Antigenic epitope composition and protectivity of avian hepatitis E virus (avian HEV) ORF2 protein and vertical transmission of avian HEV

by

Hailong Guo

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

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Ames, Iowa
2006

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DEDICATION

I wish to dedicate this thesis to my parents, my wife and daughter. To my mother, I thank you for long-lasting love. Your teaching the simple numerical computation has inspired me to study since childhood. To my father, I thank you for persistent encouragement and support throughout my years of education. Your anticipation of the first place for all courses had pushed me to go beyond the better.

To my wife, Chunfang, I thank you for supporting my idea of going to the United States for a PhD education. Without your consideration, encouragement, suggestion and sacrifice during these years, I could not have reached my goal. I specifically thank you for spending lots of time raising and educating our daughter. To my lovely daughter, Elisa, I thank you as you make me more mature as both a man and a father.
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ABSTRACT

Avian HEV was first isolated in chickens from the USA that had hepatitis-splenomegaly syndrome in 2001. Based on genetic identity and genomic organization, avian HEV has been classified into the genus *Hepevirus*, family *Hepeviridae*, which also includes human and swine HEVs. Avian HEV ORF2 protein was predicted to have common antigenic epitopes shared by avian, human and swine HEV, but its detailed antigenic epitope composition and protectivity have not been investigated. Vertical transmission of avian HEV in chickens also remains to be evaluated.

To map HEV common and non-common epitopes on avian HEV ORF2 protein, nine synthetic peptides from the predicted four antigenic domains of the avian HEV ORF2 protein were synthesized and corresponding rabbit anti-peptide antisera were generated. With the use of recombinant ORF2 proteins, convalescent pig and chicken antisera, peptides and anti-peptide rabbit sera, at least one epitope in domain II that is unique to avian HEV, one epitope in domain I that is common to avian, human and swine HEVs, and one epitope in domain IV that is shared between avian and human HEVs were identified.

To determine if the capsid ORF2 protein of avian HEV can be protective and used as vaccine, twenty chickens were immunized with purified recombinant avian HEV ORF2 protein with aluminum as adjuvant, and another twenty chickens were mock immunized with KLH precipitated in aluminum. After challenge, all the tested mock-immunized control chickens developed typical avian HEV infection but not in the tested chickens immunized with avian HEV ORF2 protein.
To identify neutralizing epitopes on avian HEV ORF2 protein, four Mabs (7B2, 1E11, 10A2, 5G10) against this protein were generated and characterized. 1E11, 10A2 and 5G10 were shown to bind to *bona fide* avian HEV particles *in vitro*, and partially neutralize virus in an animal based neutralization assay. The corresponding neutralization epitopes were further localized by Western blot with the use of five avian HEV ORF2 recombinant proteins.

Avian HEV was detected in egg white samples. Avian HEV contained in egg white was infectious as evidenced by virome, fecal virus shedding and seroconversions in the chickens inoculated with avian HEV PCR positive egg white, but not in PCR negative egg white inoculated chickens. However, vertical transmission of avian HEV in chickens was not proved.

The present studies pave the way for future avian HEV vaccine design and the development of differential immunoassays for the diagnosis of HEV cross-species infection. The results also implicate avian HEV infection in chicken could be a model system for studying HEV viral immune response, but not for studying HEV vertical transmission.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Hepatitis E virus (HEV) causes frequent endemic and rare epidemic outbreaks of acute hepatitis E disease in humans in many developing countries [1, 2]. Disease caused by HEV results in a mortality rate of about 1% in general, but in pregnant women it can reach up to 20 % due to unknown reasons [3]. Recently, increasing sporadic cases of indigenous acute hepatitis E have been reported in industrialized countries [4-12], in addition to those associated with travel to endemic areas [13-16]. Anti-HEV antibodies have also been found in up to 20% of healthy individuals in these non-endemic countries [17]. Thus, HEV infection is probably more prevalent than previously estimated.

Anti-human HEV antibodies were detected in multiple animal species [18-23]. However, to date, only two animal stains of HEV, swine HEV and avian HEV, have been isolated and well characterized [24, 25]. Swine HEV doesn’t cause clinic disease in pigs [26-28], but can cross infect humans by foodborne transmission [12, 29, 30]. Avian HEV was first isolated in the United States from chickens with hepatitis-splenomegaly (HS) syndrome [25], an emerging chicken disease in North America that is characterized by increased mortality in broiler breeder hens and laying hens between 30 and 72 weeks of age as well as up to a 20% drop in egg production [25, 31-33]. This disease was partially reproduced in SPF chickens by inoculation with avian HEV [34]. Avian HEV failed to infect non-human primates [35], but it can infect turkeys and swine [36, 37]. Based on genetic identity and genomic organization, avian HEV has been classified into the genus Hepevirus, family Hepeviridae, which also includes human and swine HEVs [38].
Three partially overlapping open reading frames (ORFs) have been identified in the HEV genome. Antibodies to all three ORF proteins appear following infection [17, 39, 40], but anti-ORF2 protein antibody is the one that can be most reliably detected in infected patients and animals [41-43]. Human HEV ORF2 recombinant protein is thus the major target antigen used in HEV serological assays [44-49]. However, it has been shown that the avian HEV recombinant ORF2 protein can be equally recognized by anti-sera raised against avian, swine and human HEVs and anti-avian HEV chicken sera can react with recombinant ORF2 proteins of avian, human and swine HEV [50]. Swine HEV ORF2 protein can also react with anti-human HEV sera and vice versa [51]. Four major antigenic domains at the C terminal 268 amino acid residues of avian HEV ORF2 protein were predicted based on the corresponding region of human HEV ORF2 proteins [50], but whether epitopes in these domains are shared with human and swine HEVs or unique to avian HEV is not known.

Human HEV ORF2 protein is the major HEV viral antigen that can elicit protective humoral immunity [52-55]. Several studies have demonstrated that immunization of truncated recombinant human HEV ORF2 protein can prevent infection by inducing efficient neutralizing antibodies [47, 55-59]. Avian HEV is genetically and antigenically related to human HEV [35, 50]. However, it is not yet known if avian HEV ORF2 protein can provide protection against avian HEV infection and if it contains neutralization epitopes that can contribute to the protection.

In addition to the primary fecal-oral route of transmission, human HEV can also be vertically transmitted from infected mothers to their fetuses resulting in significant perinatal morbidity and mortality [60-64]. Although rhesus monkeys are frequently used as an animal
model for HEV infectivity studies, pregnant monkeys failed to transmit virus to their offspring [65]. Attempts to demonstrate vertical transmission of swine HEV from pregnant gilt was also unsuccessful [66]. Therefore, it would be of interest to determine if avian HEV can be vertically transmitted and be used as a small animal model for studying some aspects of HEV vertical pathogenesis.

In order to address the problems as described above, four major objectives were developed as followed:

1. Identify antigenic epitopes on avian HEV ORF2 protein that are unique to avian HEV in addition to epitopes common to human and/or swine HEVs.

2. Evaluate the protective ability of a recombinant avian HEV ORF2 protein.


4. Investigate the possibility of vertical transmission of avian HEV in chickens.

In the first study, we defined HEV common epitopes and avian HEV unique epitopes on the avian HEV capsid protein using synthetic peptides, anti-peptide sera, ORF2 proteins of avian, human and swine HEVs and anti-HEV convalescent sera. In the second study, chickens were immunized with avian HEV ORF2 protein and challenged with avian HEV, proving that the recombinant avian HEV ORF2 protein can provide protection against avian HEV infection. In the third study, two independent neutralizing epitopes were mapped on the avian HEV ORF2 protein using monoclonal antibodies (Mabs) and serial truncated avian HEV ORF2 fragment proteins. In the fourth study, we detected avian HEV in egg whites and demonstrated that egg white avian HEV is infectious. However, we were unable to confirm that avian HEV was able to be transmitted vertically.
Overall, these results of these studies pave the way for future avian HEV vaccine design studies and the development of differential immunoassays for the diagnosis of cross-species HEV infection. Our results also demonstrated that chicken can be a small animal model for HEV immunological studies in addition to its current use for HEV pathogenesis studies, however, this model may not be suitable for HEV vertical transmission studies.

**Dissertation organization**

This dissertation is written in the alternative format and organized into seven chapters. The first chapter contains introduction of research background, statement of problems, general objectives and brief research results. The second chapter is a literature review. The next four chapters are four manuscripts that have been published, accepted, or submitted to journals. Each of these chapters is presented in the form prepared for publication. The doctoral candidate has actively participated in project writing, experiment performance, data collection and analysis, and is the senior author of all the four research papers. The first research paper titled “Identification of B-cell epitopes in the capsid protein of avian hepatitis E virus (avian HEV) that are common to human and swine HEVs or unique to avian HEV” was published in the Journal of General Virology, 2006, 87: 217-213. The second research paper, “Protection of chickens against avian hepatitis E virus (avian HEV) infection by immunization with recombinant avian HEV capsid protein”, was submitted to and accepted by Vaccine. The third research paper, “Identification of two neutralization epitopes on the capsid protein of an avian strain of hepatitis E virus by epitope mapping” was submitted to Journal of Virology. Chapter 6 is entitled “Egg whites from eggs of chickens experimentally infected with avian hepatitis E virus contain infectious virus but evidence of
complete vertical transmission is lacking”. It is the fourth research paper that was submitted to Journal of General Virology. Chapter 7 is the general conclusions and discussions for these studies as described in this dissertation. Recommendation for future research is also included in the final chapter. References are listed at the end of each chapter.

References


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CHAPTER 2. LITERATURE REVIEW

Introduction

Hepatitis E virus (HEV) is the etiological agent of hepatitis E, an acute disease that is primarily transmitted via the fecal–oral route. The disease has low mortality rates in the general population, but in pregnant women, it can result in high mortality rate, reaching approximately 20% [1]. The discovery of anti-HEV antibodies in animals and birds and the isolation and characterization of two animal HEVs, swine HEV and avian HEV [2, 3] has generated questions regarding the zoonotic potential of these viruses. Knowledge of human HEV generally exceeds what is known about animal HEV, but some aspects of research on animal HEV can provide model systems for human HEV study. In this review, the natural history, epidemiology, disease and pathogenesis, virology, immunology, diagnosis and prevention of human HEV are discussed. Brief reviews of swine HEV and avian HEV based on limited available data are also presented. A general summary of human and animal HEVs is provided at the end.

Natural history

Hepatitis E, previously known as an enterically transmitted non-A, non-B hepatitis, was first recognized in the 1980s when sera from affected patients in a waterborne viral hepatitis epidemic during 1955–56 in Delhi, India were found to lack serological markers of acute hepatitis A and B [4-6].

The first proof of the existence of a new viral hepatitis agent was obtained in 1983, when spherical 27-30 nm virus-like particles were visualized by immune electron microscopy in feces collected from a volunteer who was infected with stool material from patients with
suspected enterically transmitted non-A, non-B hepatitis [7]. This infectious virus was named as HEV in 1988 [8]. The partial genome of HEV was cloned in 1990 [9] and the full-length sequence was determined subsequently [10]. The sequencing results of the whole viral genome finally confirmed that HEV was a new virus.

The disease was also successfully transmitted to non-human primates, including different species of monkeys and chimpanzees [7, 11-14]. Non-human primates have thus been used as invaluable vectors for maintaining and passing HEV in vivo, since a cell culture system for efficient propagation of HEV is lacking.

**Epidemiology**

**Prevalence**

HEV causes epidemics of viral hepatitis in developing countries on all continents, often involving very large numbers of patients [15]. Apart from the 1955–56 New Delhi epidemic, other notable epidemics include: Kirgiz Republic, Soviet Union (1955–1956, 10 000 cases); Kathmandu Valley, Nepal (1973–1974, 10 000 cases); Mandalay, Myanmar (1976–1977, 20 000 cases); Kashmir, India (1978–1982, 52 000 cases); Xinjiang, China (1986–1988, 120 000 cases); and Kanpur, India (1991, 79 000 cases) and the Greater Darfur region, Sudan (2004, 5000 cases) [16, 17].

Sporadic cases of acute hepatitis E have also been reported in patients in developed countries such as Australia, France, Israel, The Netherlands, Spain, Italy, UK, Japan and USA [15, 18-24]. These occurrences are mainly associated with travel to endemic areas. However, recently, increasing cases of indigenous acute hepatitis E in these countries have also been reported [25-30]. Consumption of raw or undercooked shellfish, pig liver, deer and boar meat
are linked to these cases [25-27], but in some cases, the source of the infection remains unknown [28-30]. Anti-HEV antibodies have also been found in a significant number (up to 20% in some areas) of healthy individuals in areas previously thought to have low infection rates [31]. HEV infection is thus probably more prevalent in industrialized countries than previously estimated.

**Transmission**

HEV is transmitted primarily through the fecal–oral route via contaminated drinking water [32]. The disease is found most frequently in regions, where fecal contamination of the drinking water supply is common [33]. Food-borne outbreaks have been documented and believed to be the source of sporadic cases in some endemic and non-endemic areas [25-27, 34]. The occurrence of sporadic HEV infections in humans may maintain transmission during inter-epidemic periods [34]. Vertical transmission of HEV from infected mothers to their newborns has also been documented and causes significant perinatal morbidity and mortality [35-38]. Three cases of indigenous HEV infection via blood transfusion have been reported in the UK and Japan recently [39-41], suggesting hepatitis E should now be considered when investigating post-transfusion hepatitis in addition to hepatitis B and C [39]. However, person-to-person transmission of HEV is uncommon as household contact with HEV-infected patients does not appear to increase risk [32]

**Zoonotic infection**

Anti-HEV antibodies have been detected in a number of animal species including cattle, pigs, chickens, sheep, goats, nonhuman primates, deer, rodents, mongooses and
donkeys [13, 42-46]. However, to date, only two animal strains of HEVs, swine HEV and avian HEV, have been isolated and well characterized [3, 4].

Hepatitis E is a zoonotic in nature [47]. Cross-species infection by swine and human HEVs has been demonstrated. A human HEV strain has been shown to infect specific-pathogen-free (SPF) pigs and a swine HEV strain infected nonhuman primates [48, 49]. Increased risk of zoonotic HEV infection was reported in swine veterinarians and other pig workers, especially in countries where HEV is considered rare, such as in the USA and Taiwan [50]. Swine and their waste have been concluded as important sources of infection for swine veterinarians and farmers [50-52]. However, the high prevalence of anti-HEV antibodies in human populations not at apparent risk of exposure to swine HEV suggests that other sources of exposure may exist, in addition to swine HEV [50].

Most recent direct evidence of zoonotic HEV transmission came from reports from Japan, where several cases of hepatitis E occurred after patients consumed raw or undercooked livers, meats from HEV infected pigs, deer or wild boars [25-27]. The sequenced virus from the patients were identical or near identical to the viruses recovered from these animal foods.

Although one report showed that avian HEV failed to infect rhesus monkeys [53], it has been proven that avian HEV can infect turkeys [54]. And most recently, it was shown that pigs can be infected with avian HEV [55], indicating that avian HEV has the ability to adapt in some mammalian animals for replication and evolution. Currently, there is no direct evidence that avian HEV from chickens and avian HEV recovered from pigs can infect humans, however, avian HEV is still of possible zoonotic concern.
**Disease and pathogenesis**

**Disease**

Hepatitis E is a self-limiting disease with severity varying from subclinical infection to fulminant hepatitis [32, 56]. The clinical symptoms include jaundice, anorexia, hepatomegaly, abdominal pain and tenderness, nausea, vomiting and fever [32, 56-58]. HEV is usually present in the bloodstream for 1 to 2 weeks and disappears before the onset of clinical symptoms and fecal virus shedding also occurs during the incubation period and early acute phase of disease [33, 59]. However, in some infected patients, protracted viremia has been observed [60]. Symptomatic HEV infection is generally seen in middle-aged adults [32, 33]. Fulminant hepatitis E may develop and cause a low mortality rate of about 1% in the general population [32, 33]. However, the mortality rate can reach up to 20% in infected pregnant women during their 3rd trimester of pregnancy with unknown reason [36, 61-63]. There is no chronic phase of HEV infection [32, 34].

**Pathogenesis**

Most of our understanding of HEV pathogenesis came from experimental studies in non-human primates and human volunteers [64]. After the entry of HEV into the host via the oral route, the primary site of replication is probably in the intestinal tract. It is still not clear how the virus reaches the liver, but it is presumably through the hepatic portal vein [16, 65]. HEV then replicates in the cytoplasm of hepatocytes before being released into the bile and bloodstream. Liver pathology can be detected one or two weeks after virus replication, and is characterized by portal triaditis, cholestasis, lobular inflammation and degeneration of the
liver to varying degrees [16, 66-68]. Hepatic apoptosis and focal necrosis can be seen in livers with only mild inflammation [66, 67].

Serum biochemical change is strictly correlated with liver pathology. Due to the hepatocellular damage, serum alanine aminotransferase (ALT) increases dramatically at the time the virus is detected and lasts for three to four weeks [16, 69]. ALT is the representative enzyme used for the diagnosis of HEV induced acute hepatitis, although it is not a disease specific marker [66, 70]. Given that liver pathology is typically confined to small foci, it seems that the disease lesions are widespread, in order for them to be sampled by needle biopsy and detected. It also appears that the dissemination of the lesions is required to cause the sudden rise of ALT [71]. The fact that the onset of ALT elevation and the presence of histopathological changes in the liver generally correspond with the detection of anti-HEV antibody in sera and the decreasing levels of HEV antigen in hepatocytes suggests that HEV might not directly cause cytopathic changes and its pathogenesis might be mediated by the host immune responses [72].

To explain the pathogenesis of fulminant hepatitis E in pregnancy, one hypothesis put forward [72] proposes that the liver sinusoidal cells, particularly the Kupffer cells, are damaged by HEV, diminishing the ability of these cells to protect hepatocytes against endotoxins that originate from Gram-negative bacteria found in the intestinal tract. The enhanced sensitivity of pregnant women to such an endotoxin-mediated effect is well recognized and might explain the strikingly high mortality of hepatitis E in pregnancy [72]. However, the validity of this hypothesis and the precise underlying cellular and molecular mechanisms haven’t been confirmed.
Virology

Molecular Characteristics

HEV has a single-stranded, positive-sense RNA genome that is approximately 7.2 kb in size and consists of a short 5′-untranslated region (UTR), three partially overlapping open reading frames (ORFs) and a short 3′-UTR terminated by a poly(A) tract [73]. Recently, HEV was reclassified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [74]. Although the whole genome of HEV was sequenced more than 10 years ago [10], little is known about viral replication and expression *in vivo*, as HEV has not been grown reproducibly and efficiently in cell culture. Molecular biology study of HEV has focused on the three ORFs with the use of recombinant technology and infectious cDNA clone strategy [32, 75]

ORF1 encodes one polyprotein consisting of non-structural proteins that are potentially involved in viral genome replication and viral protein processing. This is suggested due to the presence of sequence motifs characteristic of viral methyltransferases, papain-like cysteineproteases, helicases and RNA-dependent RNA polymerases in the ORF1 gene [16, 76, 77]. In addition, ORF1 has two regions called Y and X domains of unknown function. Most recently, it has been shown that processing of ORF1 polyprotein is dependent upon a cysteine protease [78].

ORF2 encodes a capsid protein of 660 amino acids (aa) that may contain three glycosylation sites. The full-length ORF2 protein can be expressed as a 72 kDa protein in cultured insect cells, but quickly degrades into several subunits with the sizes of 53 kDa, 55 kDa and 63 kDa by non-viral proteolytic enzymes [79, 80]. However, the size of the ORF2
protein in native virons is not known [81]. *In vitro* assays suggest that the ORF2 protein is co-translationally translocated across the endoplasmic reticulum and is expressed intracellularly as well as on the cell surface [82]. The ORF2 protein contains important epitopes near the carboxyl ends that can induce neutralizing antibodies and has been the focus of vaccine development [83].

ORF3 is located at the end of ORF1 and overlaps with ORF1 at its 5' end by only 1 nucleotide, and overlaps with ORF2 at its 3' end by 328 nucleotides. It encodes a 123 aa protein that is expressed intracellularly and probably associates with the liver cell cytoskeleton [84] as well as facilitating virus assembly and release by interacting with nonglycoslated ORF2 protein [85]. ORF3 may also be involved in signal transduction [86, 87]. Interestingly, it has been recently shown that the ORF3 protein is required for HEV infectivity *in vivo* [88], but not for infection or production of infectious virus *in vitro* [75].

**Genotypes**

Based on phylogenetic analysis of the full-length genomes of HEV isolates, four major genotypes were grouped that also differ with respect to geographic distribution, host range, and pattern of infection [74, 89, 90]. Genotype 1 is prevalent in Asia and Africa [11, 91-93] as represented by Sar-55 from Pakistan. Virus in this genotype has been isolated only from humans and has been associated with large waterborne outbreaks. Genotype 2 contains a single strain of HEV isolated from a fecal sample collected during an outbreak of non-A, non-B hepatitis in Mexico in 1986 [94]. Genotype 3 is prevalent worldwide [89]. Viruses in this genotype that were isolated from animals (swine, deer and boar) and humans in the United States [95], the United Kingdom [96], and Japan [26, 97, 98]. Viruses of group 3 are closely
genetically related, suggesting the possibility of zoonotic transmission. Indeed, HEVs from swine, deer and boar in this genotype have caused sporadic cases of acute hepatitis in humans in industrialized countries, like Japan. Genotype 4 includes recent variant isolates from China, Japan, India, Indonesia, and Vietnam [89]. These viruses are prevalent mainly in humans and swine, but have also been reported in wild boars [99]. Viruses in this genotype are also associated with sporadic cases and food-borne outbreaks.

Although the sequences of HEV strains are very heterogeneous, it appears that all HEV isolates to date belong to a single serotype [32]. Different genotypes can’t be distinguished serologically and are also cross-protective [32, 100].

**Immunology**

**Antibody in HEV immunity**

During acute HEV infection, anti-HEV IgM precedes IgG production by a few days, appears during early phase of clinical illness and continues to be detectable up to 3 months [16]. Anti-HEV IgA can be simultaneously detected with IgM in sera during acute phase of infection [101]. However, the duration of anti-HEV IgA in serum is longer than that of anti-HEV IgM [102]. The production and role of mucosal anti-HEV IgA in infected hosts is poorly documented in the literature, although immunization of recombinant ORF2 protein can elicit an intestinal IgA response [103]. Anti-HEV IgG follows shortly after detection of IgM and peaks several weeks later, and can be detected for months and years after infection [104, 105]. Neutralization antibody plays a crucial role against HEV infection, but its dynamics in infected humans or animals hasn’t been investigated. Little is known about the anti-HEV cellular immunity.
Both active and passive immunization studies suggest that humoral immunity is the effective protective mechanism in HEV infection [66, 106-108] and antibody to the ORF2 protein is sufficient to confer protection [83, 109]. Rhesus monkeys immunized with truncated recombinant human HEV ORF2 protein expressed in insect cells can prevent HEV infection and the development of hepatitis [71, 110, 111]. Further, immunization can protect monkeys not only from homologous challenge but heterologous challenge as well [100, 110, 112, 113]. Broadly reactive anti-HEV neutralizing antibodies are induced in infected patients or immunized animals and can explain the heterologous protection [114, 115].

**Antigenic epitopes in ORF2 capsid protein**

The antigenic epitopes of the 660-aa human HEV ORF2 capsid proteins have been extensively examined with synthetic peptides and recombinant ORF2 proteins [116-119]. ORF2 antigenic epitopes are found principally located at the N and C termini, but C terminal epitopes are most important for protective immunity [118, 120, 121]. The identification of a major broadly protective neutralizing epitope on the C terminal part of human HEV Sar-55 ORF2 capsid protein [83, 114, 122] further supported this conclusion.

A full length recombinant ORF2 protein was strongly reactive with acute phase sera from HEV infected macaques and patients, but was poorly reactive with convalescent sera [117]. A partial recombinant ORF2 protein (aa394-660) was strongly reactive with both acute and convalescent phase sera and extension of this recombinant protein toward the N-terminus led to a progressive loss of convalescent phase reactivity [117, 123]. The difference in the reactivity with acute phase serum and convalescent serum between full length and partial ORF2 proteins clearly showed that the antigenic epitope profile of partial ORF2 proteins
differs and the use of partial ORF2 protein may be even better for both HEV diagnosis and vaccine development.

**Diagnosis and Prevention**

Hepatitis E can not be clinically distinguished from other types of acute viral hepatitis [124]. If outbreaks of waterborne hepatitis occur in developing countries, especially if the disease is more severe in pregnant women, or if hepatitis A is not the causative agent, hepatitis E should be considered [125]. The diagnosis of acute or fulminant hepatitis E is based on detection of the HEV genome in serum or feces by PCR assays, including RT-PCR, nested RT-PCR and recently developed real-time PCR [126-130] or detection of newly elicited anti-HEV IgA and IgM by ELISA by a commercial kit (Genelabs Technologies, Singapore) or in-house assay [101, 131, 132]. Detection of anti-HEV IgG by ELISA is most commonly used for the diagnosis of recovered HEV infection and serological HEV surveillance [133-136]. Immunofluorescent assays can be used to detect HEV antigen in liver and other tissues [137]. Detection of HEV particles in stool specimens by immune electron microscopy is infrequently used [33]. Target antigens in those serological assays are either recombinant HEV ORF2 proteins or synthetic HEV peptides that containing immunodominant epitopes of the ORF2 proteins [138-140]. One drawback of current serological diagnosis is that it can’t differentiate potential zoonotic HEV infection, as human HEV ORF2 proteins can react with anti-human, swine and avian HEV sera [141-143].

No therapeutic compounds or commercial vaccines for HEV are available yet, although one promising HEV subunit vaccine candidate, Sar-55 HEV recombinant ORF2 protein vaccine, was developed at National Institutes of Health (Bethesda, MD, USA) and is
currently undergoing a phase II/III field trial in Nepal [110]. Prevention is the most effective approach against the disease. As HEV is mainly spread by the fecal-oral route, good personal hygiene, high quality standards for public water supplies and proper disposal of sanitary waste have resulted in a low prevalence of HEV infections in many developed countries [34].

Swine HEV

**Discovery and characterization**

The first swine HEV was isolated from an HEV-seropositive commercial herd in Midwestern USA [2] after the ability of human HEV to infect swine and the presence of anti-HEV antibodies in swine was reported [144, 145]. The complete genomic sequence of swine HEV was determined and found to have a similar genomic organization to human HEV [2, 146]. Since then, swine HEV has been detected in pigs throughout the world [69]. Approximately 70-100% nucleotide sequence identity is shared between swine and human HEV [2, 147-149]. The ubiquitous nature of swine HEV infection in pigs suggests that swine are reservoirs for HEV [47].

Sequence comparison has shown that swine HEV isolates are also heterogeneous in different geographic regions and all isolates identified thus far belong to either genotype 3 or genotype 4 [52, 150]. Genotype 1 or 2 swine HEV haven’t been detected in pigs and attempts to infect pigs with genotype 1 (Sar-55) or genotype 2 (Mexico-14) strain of human HEV have been unsuccessful [150, 151], suggesting genotype 1 or 2 swine HEV may not exist and genotype 1 and 2 HEVs are restricted to humans and are not zoonotic in nature [150]. Both genotype 3 and 4 swine HEVs are associated with sporadic cases and food-borne outbreaks in
human. However, since genotype 3 swine HEV is widely distributed, evolution of virulent strains from this genotype would have more far-reaching consequences [52].

**Infection and disease**

Although swine HEV could be detected in different tissues of naturally infected pigs [152-154], unlike HEV infection in human, swine HEV infection in pigs does not cause clinical disease [48, 151]. In addition, unlike the high degree of vertical transmission of human HEV from mother to fetus, there was no vertical transmission of swine HEV and reproduction also appeared not to be affected [155]. As a result, swine HEV has limited use for HEV pathogenesis studies [156]. Currently, there is little or no demand for diagnostics or vaccination against swine HEV [69]. However, as more is learned about the zoonotic significance of swine HEV, this may change.

**Avian HEV**

**Discovery and characterization**

A study conducted in Vietnam showed that antibodies to human HEV were prevalent in 44% of tested chickens, indicating that an HEV or an HEV-like agent infected chickens [43]. In 2001, the corresponding agent was isolated in the USA from chickens that had HS syndrome [3], an emerging disease of chicken in North America [3, 157, 158]. Based on the similar genome organization of this novel chicken virus to and its genetic relatedness with mammalian HEVs (human and swine HEVs) [3, 53], it was designated avian hepatitis E virus (avian HEV) to distinguish it from mammalian HEVs.

Electron microscopic examination revealed that avian HEV is also a non-enveloped virus of 30–35 nm in diameter, with similar size and morphology to human HEV [3]. Avian
HEV shares about 50% nucleotide sequence identity with mammalian HEVs over the complete genome, 48-51% identity in ORF1, 46-48% identity in ORF2 and only 29-34% identity in ORF3 [3, 53]. Motifs in the putative functional domains of ORF1, such as the helicase and methyltransferase, were relatively conserved between avian HEV and mammalian HEVs, supporting the conclusion that avian HEV is a member of the genus Hepevirus [90].

Avian HEV was found to share common antigenic epitopes in its capsid protein with mammalian HEVs [141, 143]. Therefore, avian HEV is not only genetically but also antigenically related to mammalian HEVs. However, the importance of these epitopes in anti-avian HEV immune response hasn’t been studied yet.

Like the mammalian HEVs, cell culture for avian HEV propagation is not available. The attempts to culture it in embryonated eggs failed in one study group [159]. But, very recently, Meng, et al [160] reported the success of propagation of this virus by intravenous (IV) inoculation of 100 GE of avian HEV into embryonated chicken eggs at 9 days of embryonated age. The recovered fecal virus collected from hatched chicks can reach a titer of $10^6$ to $10^7$ GE/ml. Propagation of human and swine HEVs were also tried in this system, but was unsuccessful (X.-J. Meng, personal communication).

**Infection and disease**

Serological evidence has unequivocally shown that chickens are widely infected with avian HEV as 71% of flocks from five investigated states in USA were positive for avian HEV [161, 162]. Experimentally, avian HEV is also able to cross species barriers and infects turkeys and swine, although infected animals have not shown any clinical signs [54, 55]. The
HS syndrome was first reported in Canada in 1991 [158] and has since been recognized in the United States [157, 158]. This syndrome in US chickens is characterized by increased mortality, 20% drop in egg production in birds (primarily broiler breeders) from 30 to 72 weeks of age, presence of an enlarged liver and spleen, regressive ovaries and red fluid in the abdomen [157], whereas young birds are more often asymptomatic or subclinical [54, 162].

Avian HEV infection in chickens has been used as small animal model for HEV pathogenesis studies [156]. In experimental infection, typical liver lesions range from multifocal lesions to extensive necrosis and hemorrhage [156, 162]. Under field conditions, chickens are mostly subclinically infected, although sporadic HS syndrome has been inconsistently reproduced [156, 161]. It has been speculated that generation of sporadic disease may be dose-dependent or avian HEV infection, while an important factor, is not the sole factor for the development of clinical HS syndrome [156, 162]. Diagnosis is by observation of typical signs and lesions, and prevention is currently by thorough cleaning and disinfection after depletion of an affected flock [69].

Summary

Current human HEV research is mainly focused on molecular mechanisms of pathogenesis, vaccines and zoonosis. Swine HEV continues to be considered as important sources for sporadic cases of zoonotic human hepatitis E and a potential concern for zoonotic HEV infection in xenotransplantation with pig organs. As avian HEV is a relatively new virus and difficult to propagate in vitro, several important aspects about this virus, including pathogenesis, virus circulation and evolution in field and the detailed host immune responses need to be investigated. Since a variety of animals could be infected by HEV or HEV-like
virus, which has the potential importance as an emerging animal pathogen for public health, it is necessary to continue to search for and characterize these viruses in animals.

**References**


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CHAPTER 3. IDENTIFICATION OF B CELL EPITOPES IN THE CAPSID PROTEIN OF AVIAN HEPATITIS E VIRUS (AVIAN HEV) THAT ARE COMMON TO HUMAN AND SWINE HEVS AS WELL AS UNIQUE TO AVIAN HEV


H. Guo¹, E. M. Zhou², Z. F. Sun³, X. J. Meng³, P. G. Halbur²

Abstract

Avian hepatitis E virus (avian HEV) was recently discovered in chickens from the USA that had hepatitis–splenomegaly (HS) syndrome. The complete genomic sequence of avian HEV shares about 50% nucleotide sequence identity with those of human and swine HEVs. The open reading frame 2 (ORF2) protein of avian HEV has been shown to cross-react with human and swine HEV ORF2 proteins, but the B-cell epitopes in the avian HEV ORF2 protein have not been identified. Nine synthetic peptides from the predicted four antigenic domains of the avian HEV ORF2 protein were synthesized and corresponding rabbit anti-peptide antisera were generated. Using recombinant ORF2 proteins, convalescent pig and chicken antisera, peptides and anti-peptide rabbit sera, at least one epitope at the C terminus

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of domain II (possibly between aa 477–492) that is unique to avian HEV, one epitope in
domain I (aa 389–410) that is common to avian, human and swine HEVs, and one or more
epitopes in domain IV (aa 583–600) that are shared between avian and human HEVs were
identified. Despite the sequence difference in ORF2 proteins between avian and mammalian
HEVs and similar ORF2 sequence between human and swine HEV ORF2 proteins, rabbit
antiserum against peptide 6 (aa 389–399) recognized only human HEV ORF2 protein,
suggesting complexity of the ORF2 antigenicity. The identification of these B-cell epitopes in
avian HEV ORF2 protein may be useful for vaccine design and may lead to future
development of immunoassays for differential diagnosis of avian, swine and human HEV
infections.

Introduction

Hepatitis E virus (HEV) causes frequent endemic and rare epidemic outbreaks of
acute hepatitis E in many developing countries. Sporadic cases of acute hepatitis E have also
been reported in industrialized countries including the USA, Europe and Japan (Clemente-
Casares et al., 2003; Mansuy et al., 2004; Okamoto et al., 2003). HEV is transmitted by
faecal contamination of water. Cross-species infection has been documented as human and
swine HEV strains are genetically closely related, and experimental cross-species infection of
swine HEV to a chimpanzee and human HEV to pigs has been demonstrated (Meng et al.,
1998, 2002; Meng, 2003; Halbur et al., 2001). It is now recognized that hepatitis E is a
zoonosis, and pigs and rats are considered to be the reservoir for HEV (Hirano et al., 2003;
Kabrane-Lazizi et al., 1999; Meng et al., 1997, 1999b; Meng, 2005).
Hepatitis–splenomegaly (HS) syndrome is an emerging disease of chicken in North America (Haqshenas et al., 2001; Riddell, 1997; Ritchie & Riddell, 1991). Avian hepatitis E virus (avian HEV) was isolated from chickens in the USA in 2001 (Haqshenas et al., 2001) that had HS syndrome, and lesions characteristic of HS syndrome have recently been reproduced in specific-pathogen-free (SPF) chickens (Billam et al., 2005). A recent study indicated that avian HEV is heterogenic and enzootic in chicken flocks as some avian HEV strains spread subclinically among chickens in the USA (Huang et al., 2002; Sun et al., 2004a).

Avian HEV is a member of the genus *Hepevirus* (Emerson et al., 2004), which also includes human and swine HEVs. Phylogenetic analysis revealed that avian HEV represents a branch distinct from human and swine HEVs (Huang et al., 2004). The genome of mammalian HEV is approximately 7.2 kb in size and contains three open reading frames (ORFs) (Emerson & Purcell, 2003). ORF1 encodes viral non-structural proteins, ORF2 encodes the putative capsid protein and ORF3 encodes a small protein that may be involved in modulating cell signalling (Emerson & Purcell, 2003; Meng et al., 1999a), suggesting a possible role in host–virus interaction. The genome of avian HEV is about 600 nt shorter than those of swine and human HEVs (Huang et al., 2004). Humoral immune response is important for protection, and the ORF2 capsid protein is immunogenic and is thought to be responsible for the induction of humoral immune responses (Zhang et al., 2001; Riddell et al., 2000). Thus, the ORF2 capsid protein is the target of current vaccine design and its recombinant proteins expressed either in *Escherichia coli* or insect cells are used for evaluation of vaccine efficacy (Purcell et al., 2003). It has been demonstrated that
chimpanzee antibodies specific for linear epitopes in the ORF2 capsid protein neutralized human HEV infection in rhesus monkeys (Schofield et al., 2000, 2003).

The ORF2 gene of avian HEV shares approximately 48–49 % amino acid sequence identity with that of swine HEV and the US2 and Sar-55 strains of human HEV (Haqshenas et al., 2002). Four major antigenic domains at the C-terminal 268 aa residues of capsid protein from avian, swine and human HEV were predicted by Haqshenas et al. (2002) according to the methods of Kyte–Doolittle and Welling using the MacVector computer program (Oxford Molecular) and based on the hydrophilicity and antigenicity. Domain I is most conserved among avian, human and swine HEVs, whereas domain IV is more antigenic in avian HEV than in swine and human HEVs.

In the present study, we demonstrated that B-cell epitopes in the antigenic domain I are shared among avian, human and swine HEV capsid proteins, and that epitopes in domain IV are shared between avian and human HEVs. However, epitopes in domain II are unique to avian HEV.

Materials and Methods

Production of recombinant avian HEV ORF2 protein. The expression, extraction and purification of ORF2 protein were performed essentially as described previously (Haqshenas et al., 2002) with the following modifications. After initial purification by using His-Bind resin column (Novagen), the ORF2 protein was further purified by electroelution. Briefly, ORF2 inclusion body protein effluent in the elution buffer (Novagen) suspension was mixed with the reducing Laemmli sample buffer (Bio-Rad) and loaded onto 7·5 % SDS-PAGE gels and proteins were subsequently separated. After washing with distilled water, the
gels were negatively stained with copper stain buffer (Bio-Rad). The corresponding 32 kDa ORF2 protein band was excised and the copper stain was removed using the destain buffer (Bio-Rad). The destained gel slices containing ORF2 protein were subjected to electroelution using a commercial apparatus (Bio-Rad). Eluted proteins were collected and the concentration was determined using a Protein assay kit (Bio-Rad). The purity of eluted proteins was confirmed by SDS-PAGE with Coomassie brilliant blue R-250 (Bio-Rad). The purified ORF2 proteins were used in the ELISA.

**Swine and chicken convalescent serum samples.** Swine convalescent serum samples were collected from pigs experimentally infected with swine HEV and human HEV as described previously (Halbur *et al.*, 2001). Chicken convalescent serum samples were collected from chickens experimentally infected with avian HEV (Sun *et al.*, 2004b). The experimental swine and chicken serum samples used in this study were collected at 28 and 42 days post-infection, respectively. A panel of positive and negative swine serum samples from naturally infected pigs from different countries (Meng *et al.*, 1999b) was also used in this study.

**Peptide synthesis and production of rabbit antisera.** Nine truncated peptides were commercially synthesized and purified (SynPep). The amino acid sequences of the peptides and their locations on avian HEV ORF2 protein are shown in Fig. 1. Peptides 1, 2, 3 and 4 include the full-length of domains I, II, III and IV, respectively. Peptides 5 and 6 contain the second and first half of domain I, respectively; peptides 7 and 8 consist of the first and second half of domain II, respectively; and peptide 9 has four more amino acid residues than peptide 8 at the N terminus. All peptides were conjugated with keyhole limpet haemocyanin (KLH)
and each peptide-KLH was emulsified with an equal volume of Freund's complete adjuvant. Each peptide (100 µg per rabbit) was used to immunize two New Zealand white rabbits. The initial immunization was given on day 0 by intramuscular injection. A booster injection with peptide-KLH in Freund's incomplete adjuvant was given 14 days later. Blood was collected before each injection and 14 days after the booster.

**Detection of rabbit anti-peptide antibodies.** Two rabbit sera from each peptide-immunized group were tested immediately after each bleed and pooled. The pooled sera from two rabbits against peptide 1 through to peptide 9 were designated RS1–RS9 and used throughout this study. Each of the pooled rabbit sera was tested by an ELISA against each of the nine peptides as well as avian HEV ORF2 protein, human HEV (Sar-55 strain) recombinant ORF2 protein (Robinson *et al.*, 1998) and swine HEV recombinant ORF2 protein (Meng *et al.*, 2002). ELISA plates (Nunc) were coated with peptides or ORF2 antigens at 100 ng per well overnight at 4 °C. After washing with PBS/T [0·01 M PBS, pH 7·2, containing 0·05 % Tween 20 (v/v)], the wells were blocked with 3 % BSA (w/v) in PBS/T for 60 min at room temperature. Rabbit sera at various dilutions in PBS/T were added in duplicate and incubated for 60 min at room temperature. Goat anti-rabbit immunoglobulin G (IgG) (H+L)-HRP conjugate (Jackson ImmunResearch) (diluted 1/1000 in PBS/T, 100 µl per well) was added to each well. After incubating for 60 min and a final washing step, the substrate (*O*-phenylenediamine dihydrochloride; Sigma-Aldrich) was added for colour development. After 15 min the reaction was stopped by adding 3 M H₂SO₄ to each well (50 µl per well), and read at *A*_490 on an automatic ELISA plate reader (Universal Microplate Reader, EL800; Bio-Tek Instrument).
Detection of swine or chicken antibodies against ORF2 antigen and peptides.

Swine or chicken anti-HEV antibodies were detected using ELISA as described above except that the sera were diluted 1/100 in PBS/T, goat anti-swine IgG (H+L) and goat anti-chicken IgY-HP conjugate (Jackson ImmunResearch) were used as the secondary antibodies.

Statistical analysis. Statistical analysis was performed using t-test (Microsoft Excel 2003) to compare the differences in ELISA absorbance values between pre-immune and immune rabbit sera reacting with ORF2 antigens. P values of ≤ 0.05 were considered significant.

Results

Expression and purification of avian HEV ORF2 protein

As expected, the avian HEV ORF2 protein was expressed as an inclusion body in bacterial cells with a high yield (Fig. 2a and b). Further purification of the ORF2 protein was performed by electroelution. From 9 mg of inclusion body effluent proteins, which originated from 100 ml of bacterial culture, we obtained approximately 5.4 mg of purified ORF2 proteins, as demonstrated in the SDS-PAGE gel (Fig. 2c).

Titration of rabbit antisera

Titration of rabbit anti-peptide antisera (RS1–RS9) was performed by ELISA. Serum titres were defined as the highest serum dilution that gave an $A_{490}$ of 1.0. RS2–RS7 had titres between $10^3$ and $10^4$ as shown in Fig. 3, and RS1, RS8 and RS9 had titres between $10^4$ and $10^5$. The titres of pre-immune rabbit sera were <$10^2$ (data not shown).

Cross-reactivity of rabbit antisera with nine synthetic peptides
To determine the cross-reactivity of rabbit antisera against the nine peptides, RS1–RS9 were tested against individual peptides in the ELISA. Three peptides (peptides 1, 5 and 6) were generated from domain I. As shown in Fig. 4, RS1 reacted with peptides 1 and 5 ($A_{490}$ 1.280 and 1.111, respectively) and RS5 reacted with peptides 5 and 1 ($A_{490}$ 0.810 and 1.213, respectively). RS1 and RS5 did not cross-react with peptide 6. RS6 reacted only with peptide 6 but not with peptides 1 and 5. These data suggested that the B-cell epitope(s) of domain I was located in the second half of the domain (aa 400–410).

For domain II, four peptides (peptides 2, 7, 8 and 9) were produced and used to immunize rabbits. RS2 reacted with peptides 2 ($A_{490}$ 1.136), 8 ($A_{490}$ 1.026) and 9 ($A_{490}$ 1.239) but not with peptide 7 ($A_{490}$ 0.062). RS8 and RS9 cross-reacted with peptide 2 ($A_{490}$ 0.631 and 0.617, respectively) and to each other ($A_{490}$ 1.020 and 1.185, respectively). In contrast, RS7 only reacted with peptide 7 ($A_{490}$ 0.964) and did not react with peptide 2 ($A_{490}$ 0.130). These results suggest that one or more B-cell epitopes are located in the second half of domain II (aa 477–492) and that the N-terminal 4 aa extension in peptide 9 is an important part of the epitope. As expected, there was no cross-reaction among peptides 1, 2, 3 and 4.

Cross-reactivity of rabbit antisera against ORF2 proteins of avian, swine and human HEVs

To determine whether rabbit anti-peptide sera can recognize ORF2 proteins, RS1–RS9 were tested for their reactivity with avian, swine and human HEV recombinant ORF2 antigens by ELISA. As shown in Fig. 5, in comparison with pre-immune sera, RS1, RS4, RS8 and RS9 reacted with avian HEV ORF2 ($P$ values of 0.02, 0.004, 0.02 and 0.01, respectively). RS1 along with RS4 and RS6 also reacted with human HEV ORF2 ($P$ value of
0·002, 0·02 and 0·01, respectively). Only RS1 reacted with swine HEV ORF2 (\(P\) value of 0·02). Thus, RS1 reacted with all three recombinant ORF2 antigens.

A panel of swine serum samples from pigs naturally infected with swine HEV (Meng et al., 1999b) was also used to evaluate the cross-reactivity with avian HEV ORF2 antigens. Three swine HEV antibody-positive pig sera from Canada reacted with avian HEV ORF2 antigen, but only one reacted with peptide 1 (Table 1), whereas three negative sera did not react with these antigens. Similar results were also obtained from swine HEV-positive pig sera from Korea, Thailand and the USA except that one pig from Korea and one from Thailand reacted with peptide 4 in addition to avian HEV ORF2 antigen and peptide 1. The third pig serum from Thailand reacted with avian HEV ORF2 antigen and peptide 1. Three pig sera from China reacted with avian HEV ORF2 antigen and peptides 1–4, suggesting that these pigs were infected with avian HEV, since pigs and chickens are reared together in many backyard farms in China. This may indicate the possibility of transmission of avian HEV from chicken to pigs. This possibility was further supported by the fact that pigs can be experimentally infected with avian HEV as reported by Kasorndorkbua et al. (2005).

As positive and negative controls, serum samples from six chickens experimentally infected with avian HEV and four chickens negative for avian HEV were tested against these antigens. Convalescent serum samples collected at 42 days post-inoculation strongly reacted with avian HEV ORF2 antigen and peptide 1, 4 and 8 (Table 1) but not with peptides 2 and 3. Collectively, these data indicate that the B-cell epitopes present in the antigenic domain I are common to avian, human and swine HEV ORF2 proteins. A B-cell epitope in domain II might be unique to avian HEV. Although the B-cell epitopes in domain IV are shared
between avian and human HEVs, the fact that pig sera from swine HEV naturally infected pigs collected from China, Korea and Thailand suggests the possible transmission of avian HEV to pigs.

**Discussion**

Avian HEV is an emerging virus that is enzootic in chicken flocks in the USA. Current ELISA methods using the recombinant ORF2 protein for serological diagnosis cannot differentiate avian HEV from human and swine HEV infections due to the cross-reactivity between the ORF2 capsid antigens (Haqshenas *et al.*, 2001). Based on the four predicted major antigenic domains in avian HEV ORF2 capsid protein (Fig. 1), we synthesized nine peptides and produced rabbit antisera against them (Fig. 2) to identify epitopes that are specific for avian HEV and that are common to human and swine HEVs.

Cross-reactivity studies using the rabbit antisera against the nine peptides revealed that a B-cell epitope is located in the second half of domain I since RS1 (against peptide 1, entire domain I between aa 389 and 410) and RS5 (against peptide 5, between aa 399 and 410) cross-reacted with each other (Fig. 4). We speculate that there may be a B-cell epitope in domain I between aa 399 and 410, since RS6 (against peptide 6, aa 389–398) did not cross-react with peptide 1, and peptide 5 is only 12 aa long. However, cross-reactivity studies between rabbit antisera and recombinant ORF2 proteins suggested that additional epitopes may exist in domain I that are common among avian, human and swine HEV ORF2 proteins, since RS1 reacted with all three ORF2 proteins (Fig. 5). The facts that RS1 reacted more strongly with human ($A_{490} > 3.0$) and swine HEV ORF2 proteins ($A_{490} 1.5$) than with avian HEV ORF2 protein ($A_{490} 0.82$), and that RS5 did not react with any ORF2 proteins and RS6
reacted only with human HEV ORF2 suggest that these epitopes in domain I may be conformational and presented differently among these three ORF2 proteins, and thus may be influenced by amino acid residues outside these domains. This was further supported by the fact that, despite a 98 % amino acid sequence identity between human ORF2 (Sar-55) and swine ORF2 (Meng et al., 1999b), RS6 only reacted with human HEV ORF2 and not with swine HEV ORF2 proteins.

Domain II is the longest of the four predicted antigenic domains, with 32 aa (aa 461–492); therefore, four peptides were synthesized with 32, 16, 16 and 20 aa, corresponding to peptides 2, 7, 8 and 9, respectively (Fig. 1). Rabbit antisera of RS2, RS8 and RS9 cross-reacted with each of the others, indicating that one or more B-cell epitopes on avian HEV ORF2 are located between aa 473 and 492 at the C terminus. Since RS8 and RS9 only reacted with avian HEV ORF2 (Fig. 5) and since they cross-reacted equally well with each other and with peptide 2 (Fig. 4), the epitope on the antigenic domain II is likely to be located between aa 477 and 492 and expressed only on avian HEV ORF2 protein (Fig. 4). The fact that RS2 did not react with avian HEV ORF2 suggested that the N-terminal amino acid residues may block the C-terminal epitope presentation, since peptides 8 and 9 are truncated from the C terminus of peptide 2 (Fig. 1). This result is consistent with the findings by Riddell et al. (2000) in which the monoclonal antibodies did not recognize human HEV ORF2 antigen within this region, indicating that other domains are involved in the antigenicity of domain II.

Antigenic cross-reactivity studies using antisera from pigs naturally infected with swine HEV and experimentally infected with swine and human HEVs (Table 1) support the hypothesis that avian HEV ORF2 domain I contains at least one epitope that is common
among avian, human and swine HEVs and domain IV contains epitope(s) shared between
avian and human HEVs. The epitopes in domain IV that are shared between avian and human
HEVs are likely to be located at the C terminus of ORF2 (Schofield et al., 2003), despite the
sequence differences between these proteins (Fig. 1). In contrast, peptide 2 contains a B-cell
epitope only presented on avian HEV ORF2, as demonstrated by the fact that RS8 and RS9
rabbit antisera reacted with avian HEV ORF2 (Fig. 5) and that convalescent antisera from
chickens experimentally infected with avian HEV also reacted with peptide 8 (Table 1).
Interestingly, convalescent chicken antisera did not react with peptide 2 but did react with
peptide 8, suggesting that the epitope in peptide 8 was not accessible on peptide 2 when it was
used in ELISA.

In summary, we found that domains I and IV contained epitopes in ORF2 protein that
are shared among three strains of HEV, supporting further the classification of avian HEV in
the genus *Hepevirus* of the family *Hepeviridae* (Emerson et al., 2004). ORF2 protein from
either avian, human or swine HEV can be used to detect anti-HEV antibodies. The ORF2
proteins from human and swine HEVs have been shown to be equally efficient as diagnostic
reagents for the detection of anti-HEV antibodies (Engle et al., 2002). In this study, we found
that peptide 4, corresponding to antigenic domain IV, is useful for the detection of avian and
human HEVs and that peptide 8 can be used for the detection of anti-avian HEV antibodies.
Currently, we do not know if these epitopes are neutralizing epitopes. Future studies of the
immunogenicity of peptides 1, 4 and 8 along with avian HEV ORF2 protein in chickens and
their ability to protect against avian HEV infection are warranted.
Acknowledgements

We thank Drs Robert Purcell and Suzanne Emerson at the National Institute of Health, Bethesda, MD for providing the purified recombinant Sar-55 human HEV and swine HEV recombinant ORF2 proteins. Part of this work is supported by a grant (to X.-J. M.) from the National Institute of Health (AI 50611).

References


Table 1. Antigenic cross-reactivity of convalescent antisera from pigs experimentally infected with swine and human HEVs, from pigs naturally infected with swine HEV and from chickens experimentally infected with avian HEV with truncated ORF2 protein and five synthetic peptides of avian HEV. Indirect ELISA was used to detect swine and chicken sera against ORF2 and peptides. The sera were diluted at 1/100 and the ORF2 and peptides were used at 100 ng per well.

<table>
<thead>
<tr>
<th>No. samples</th>
<th>Source</th>
<th>ELISA results against avian HEV ORF2 and five peptides*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ORF2                                            Peptide 1</td>
</tr>
<tr>
<td>Convalescent swine antisera from experimentally infected pigs</td>
<td></td>
<td>1.186 ± 0.1041</td>
</tr>
<tr>
<td>2</td>
<td>Negative sera</td>
<td>1.012 ± 0.004</td>
</tr>
<tr>
<td>3</td>
<td>Swine HEV</td>
<td>1.747 ± 0.208</td>
</tr>
<tr>
<td>6</td>
<td>Human HEV</td>
<td>1.186 ± 0.1041</td>
</tr>
</tbody>
</table>

Antiserum from naturally infected pigs

<table>
<thead>
<tr>
<th>No. samples</th>
<th>Source</th>
<th>ORF2</th>
<th>Peptide 1</th>
<th>Peptide 2</th>
<th>Peptide 3</th>
<th>Peptide 4</th>
<th>Peptide 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Canada, negative</td>
<td>0.104 ± 0.003</td>
<td>0.960 ± 0.003</td>
<td>0.262 ± 0.019</td>
<td>0.003 ± 0.017</td>
<td>0.079 ± 0.019</td>
<td>0.041 ± 0.005</td>
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<tr>
<td>1</td>
<td>Canada, positive</td>
<td>1.148</td>
<td>0.574</td>
<td>0.106</td>
<td>0.103</td>
<td>0.141</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>Canada, positive</td>
<td>1.232 ± 0.063</td>
<td>0.188 ± 0.014</td>
<td>0.330 ± 0.021</td>
<td>0.110 ± 0.013</td>
<td>0.131 ± 0.009</td>
<td>0.045 ± 0.004</td>
</tr>
<tr>
<td>3</td>
<td>China, negative</td>
<td>0.152 ± 0.011</td>
<td>0.194 ± 0.029</td>
<td>0.334 ± 0.017</td>
<td>0.135 ± 0.009</td>
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<td>0.045 ± 0.004</td>
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<tr>
<td>3</td>
<td>China, positive</td>
<td>2.814 ± 0.112</td>
<td>0.641 ± 0.016</td>
<td>0.467 ± 0.103</td>
<td>0.419 ± 0.124</td>
<td>0.608 ± 0.171</td>
<td>0.043 ± 0.001</td>
</tr>
<tr>
<td>4</td>
<td>Korea, negative</td>
<td>0.192 ± 0.022</td>
<td>0.083 ± 0.020</td>
<td>0.075 ± 0.012</td>
<td>0.082 ± 0.023</td>
<td>0.081 ± 0.018</td>
<td>0.049 ± 0.008</td>
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<tr>
<td>1</td>
<td>Korea, positive</td>
<td>0.448</td>
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<td>0.100</td>
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<tr>
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<td>Korea, positive</td>
<td>0.973</td>
<td>0.458</td>
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<td>0.136 ± 0.009</td>
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<td>0.119 ± 0.065</td>
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Convalescent antiserum from chickens experimentally infected with avian HEV

<table>
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<th>No. samples</th>
<th>Source</th>
<th>ORF2</th>
<th>Peptide 1</th>
<th>Peptide 2</th>
<th>Peptide 3</th>
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<th>Peptide 8</th>
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<td>2</td>
<td>Pre-sera</td>
<td>0.183 ± 0.010</td>
<td>0.054 ± 0.009</td>
<td>0.023 ± 0.007</td>
<td>0.084 ± 0.001</td>
<td>2.322 ± 0.434</td>
<td>0.000 ± 0.001</td>
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<tr>
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<td>Positive</td>
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<td>0.069 ± 0.013</td>
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<td>2.322 ± 0.434</td>
<td>0.125 ± 0.125</td>
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* The numbers are the mean ELISA absorbance values ± standard deviation.
† The values in bold are considered positives.
Figure 1. Amino acid sequence and locations of 9 synthesized peptides in avian HEV ORF2 protein (Haqshenas, et al., 2002). (a) Peptides 1, 2, 3, 4 are the full antigenic domain I, II, III, IV, respectively; peptides 5 and 6 are truncated from peptide 1; peptides 7, 8, 9 are derived from peptide 2. (b) Amino acid sequences in the four antigenic domains were compared to the corresponding region of swine HEV and human HEV (Sar-55 strain). Dots represent residues identical to those in avian HEV. Dashes represent amino acid residues deletions in swine and human HEV.
Figure 2. Expression and purification of avian HEV ORF2 protein. (a) Lanes 1-2, SDS-PAGE analysis of two bacterial cell clone lysates at 4 hours after IPTG induction. (b) Lanes 1-3, SDS-PAGE analysis of three continuous effluent fractions from inclusion body purification with His-Bind resin column. (c) Lane 1, SDS-PAGE analysis of inclusion body proteins after electric elution purification. The expected size of the truncated avian HEV ORF2 proteins product is 32kDa.
Figure 3. Titration of rabbit anti-sera (RS1–9). Each point represents the mean absorbance values obtained from RS1–9 performed in duplicates. The absorbance value of 1·0 was used as the end-point titre.
Figure 4. Cross-reaction of rabbit anti-sera (RS1-9) with nine peptides. Rabbit sera were diluted at 1/1000 and used in the ELISA against peptides 1-9 (from left to right in each panel column). Each bar represents the mean absorbance values obtained from RS1-9 performed in duplicates ± standard error.
Figure 5. Reaction of rabbit anti-sera (RS1–9) with avian HEV ORF2 (black), human HEV ORF2 (grey) and swine HEV sORF2 (hatched) antigens. Open bars, pre-immune sera. Each bar represents the mean absorbance values obtained from RS1–9 performed in duplicates ± standard error. All sera were diluted at 1/1000. *, Statistical significance compared with the pre-immune sera ($P \leq 0.05$).
CHAPTER 4. PROTECTION OF CHICKENS AGAINST AVIAN HEPATITIS E VIRUS (AVIAN HEV) INFECTION BY IMMUNIZATION WITH RECOMBINANT AVIAN HEV CAPSID PROTEIN

A paper accepted by Vaccine 2006†

H. Guo¹, E. M. Zhou², Z. F. Sun³, X. J. Meng³

Abstract

Avian hepatitis E virus (avian HEV) is an emerging virus associated with hepatitis-splenomegaly syndrome in chickens in North America. Avian HEV is genetically and antigenically related to human HEV, the causative agent of hepatitis E in humans. In the lack of a practical animal model, avian HEV infection in chickens has been used as a model to study human HEV replication and pathogenesis. A 32 kDa recombinant ORF2 capsid protein of avian HEV expressed in E. Coli was found having similar antigenic structure as that of human HEV containing major neutralizing epitopes. To determine if the capsid protein of avian HEV can be used as a vaccine, twenty chickens were immunized with purified avian HEV recombinant protein with aluminum as adjuvant, and another twenty chickens were mock immunized with KLH precipitated in aluminum as controls. Both groups of chickens were subsequently challenged with avian HEV. All the tested mock-immunized control

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chickens developed typical avian HEV infection characterized by viremia, fecal virus shedding and seroconversion to avian HEV. Gross hepatic lesions were also found in portion of these chickens. In contrast, none of the tested chickens immunized with avian HEV capsid protein had detectable viremia, fecal virus shedding or observable gross hepatitis lesions. The results from this study suggested that immunization of chickens with avian HEV recombinant ORF2 capsid protein with aluminum as adjuvant can induce protective immunity against avian HEV infection. Chickens are a useful small animal model to study anti-HEV immunity and pathogenesis.

**Key words:** Avian hepatitis E virus; ORF2 capsid protein; Immunization.

**Abbreviated article title:** Protection of chickens against avian HEV infection.

**Introduction**

Avian hepatitis E virus (HEV) was recently isolated from chickens with hepatitis-splenomegaly syndrome (HS) syndrome in the United States [1] and proved to be the primary causative agent of HS syndrome [2], an emerging chicken disease in North America [1,3,4]. Avian HEV is a member of the genus *Hepevirus*, which also includes human and swine hepatitis E viruses [5]. Phylogenetic analysis revealed that avian HEV represents a branch distinct from human and swine HEVs, and probably represents a 5th genotype [6]. The genome of mammalian HEVs is approximately 7.2 kb in size and contains three partially overlapping open reading frames: ORF1, ORF2, and ORF3 [7] which encode, respectively, for nonstructural proteins, putative capsid protein, and a small protein that may be involved in HEV structural assembly and virus-host interaction [8–10]. The genomic organization of avian HEV is similar to that of mammalian HEVs, although avian HEV is approximately 600
bp shorter than mammalian HEVs because of the majority of the deletions in the hypervariable region of avian HEV ORF1 [6].

Humoral immune responses play an important role in anti-HEV immunity, and are mostly induced by the ORF2 capsid protein [11–15]. Several studies have demonstrated that the ORF2 capsid protein can induce efficient neutralizing antibodies to prevent HEV infection [16–20]. Rhesus monkeys immunized with truncated human HEV ORF2 protein expressed in insect cells can avoid HEV infection and development of hepatitis [21–24]. The human HEV recombinant ORF2 protein containing the major neutralizing epitopoes is currently used as a vaccine in a field trial in Nepal [25]. Immunization with recombinant HEV ORF2 protein can protect monkeys not only from homologous virus challenge but heterologous challenges as well [20, 26,27 and 28]. Human HEV ORF2 proteins expressed in bacteria were equally efficient in eliciting protective immunity against HEV infection [29,30]. Human HEV ORF2 DNA vaccines also induced protective anti-ORF2 antibody responses [31–33].

We reported the expression of the C-terminal 268 residues of avian HEV ORF2 protein in bacterial cells and demonstrated that it contains similar antigenic domains to the corresponding region of human and swine HEVs and shares common antigenic epitopes with that of human and swine HEVs, although avian HEV capsid protein is approximately 60 amino acids shorter than the mammalian HEV counterparts [6,34]. Two B cell epitopes common to both avian HEV and human HEV were recently identified on the avian HEV capsid protein [35]. In this study, we further demonstrated that the recombinant ORF2 protein of avian HEV can provide protective immunity against avian HEV infection in
Materials and Methods

2.1. Avian HEV virus

Since avian HEV can not be propagated in vitro [36], an avian HEV infectious stock was produced by intravenously inoculating chickens with 200 μl of the avian HEV fecal suspensions with a titer of $10^3$ 50% chicken infectious doses (CID$_{50}$) per ml [36]. The generated avian HEV stock containing $1 \times 10^4$ genome equivalent (GE)/ml or 500 CID$_{50}$/ml of avian HEV in 10% feces suspension was used for infecting chickens.

2.2. Specific-pathogen-free (SPF) chickens

Forty 15-week-old SPF chickens purchased from M&C Anderson Pullets (Sioux Rapids, IA) and tested negative for anti-avian HEV ORF2 antibody were divided into two groups of 20 each and housed in two separated rooms. Group 1 chickens (ID #25 – #44), four chickens per cage, were each immunized with avian HEV ORF2 protein, and group 2 chickens (ID #5 – #24), four chickens per cage, were mock immunized as controls.

2.3. Immunization of chickens with avian HEV ORF2 protein

The recombinant pRSET-C plasmid containing the truncated ORF2 gene of avian HEV was constructed as described previously [34]. The expression and purification of the truncated avian HEV ORF2 protein were described elsewhere [35,36]. The purified avian HEV ORF2 protein was coupled to Keyhole Limpet Hemocyanin (KLH) (Pierce, Rockford, IL) and precipitated in aluminum according to the protocol established previously [37]. Chickens in group 1 were each immunized intramuscularly with 200 μg of purified avian HEV ORF2 protein at two sides of the breast, and boosted twice at two weeks intervals.
Chickens in group 2 were each similarly given equal amounts of KLH precipitated in aluminum. Blood was collected from all chickens prior to immunization, and weekly after immunization from each chicken.

2.4. Virus challenge and sample collection

Two weeks after the second booster immunization, all chickens were challenged intravenously with 100 µl of the avian HEV infectious stock containing 1000 GE or 50 CID<sub>50</sub> virus dose. Serum and fecal samples were collected weekly from each chicken and used for detection of viremia and the presence of avian HEV RNA. Fecal virus shedding and viremia were monitored for 5 weeks. Four chickens from each group were necropsied at one, two, four weeks post-challenge (WPC), respectively, and the remaining chickens were necropsied at five WPC. During each necropsy, bile samples were collected from each chicken and gross liver pathological changes were recorded by digital camera.

2.5. Detection of avian HEV by RT-PCR

To detect avian HEV RNA by RT-PCR, the viral RNA was extracted from 100 µl of fecal, serum or bile samples with with RNAgent<sup>®</sup> Total RNA Isolation System (Promega). Total RNA was resuspended in 10 µl of DNase-free, RNase-free and proteinase-free water (MP Biomedicals, CA). Reverse transcription (RT) was performed at 42 °C for 60 min in the presence of a master mixture consisting of 10 µl of total RNA, 0.25 µl of AMV reverse transcriptase (Promega), 1 µl of 10 µM antisense primer, 0.5 µl of RNase inhibitor, 4 µl of 5 × RT buffer, and 1 µl of 10 mM dNTP.

The resulting cDNA was amplified by a nested RT-PCR with Taq DNA polymerase (Promega) and two pairs of primers as described previously [36] with the following
modifications. For the first round PCR, the reactions were incubated at 95 °C for 5 min, followed by 38 cycles of amplification at 94 °C for 1 min for denaturation, 1 min at 52 °C for annealing, and 1 min at 72 °C for extension, with a final incubation period at 72 °C for 7 min. For the second round PCR, 5 µl of the first PCR products were used as the templates. The parameters were the same as the first round PCR with the exception of 53 °C annealing temperature and 35 amplification cycles. PCR was performed using the Mastercycler Personal (Eppendorf, NY) thermocycler. The final PCR products were examined on a 1.2% agarose gel containing with 1.0 µg/ml ethidium bromide.

2.6. Sequencing and sequence analysis

Two positive fecal samples were selected and amplified by RT-PCR using the high-fidelity ProofStart DNA polymerase (Qiagen, CA). The final PCR products of 221bp were sequenced for both strands using ABI 3730 DNA Analyzer (Applied Biosystems, CA) at Iowa State University DNA Facility (Ames, Iowa) to confirm the identity of the viruses recovered from the infected chickens. Sequences were aligned and analyzed using the MegaAlign program (DNA STAR Inc., Madison, WI).

2.7. ELISA assay

Chicken serum samples collected 2 weeks after 2nd and 3rd immunization and weekly after virus challenge were titrated by ELISA to determine the anti-avian HEV ORF2 antibody levels. Briefly, 96-well flat bottom plates (Nunc™, USA) were coated with avian HEV ORF2 proteins (100 ng/well) overnight at 4°C. Each well of the plate was blocked with 200 µl of 2.5% non-fat dry milk in PBS containing 0.05% Tween-20 (PBS’T) for 1 hour at room temperature. After washing with PBS’T, chicken sera diluted in 2.5% non-fat dry milk
PBS'T were added to the plate in duplicates and incubated for 1 hour at room temperature. Goat anti-chicken IgY Fc fragment-HRP conjugate (Bethyl Laboratories Inc., Texas) (1:2000 diluted in PBS'T, 100 µl/well) were added to each well and incubated for 1 hour. After a final washing step, 100 µl of o-Phenylenediamine Dihydrochloride substrate (Sigma) was added to develop colorimetric reaction for 15 min. The reaction was then stopped by adding 3M H₂SO₄ in each well (50 µl/well) and read at 490 nm on an automatic ELISA plate reader (Universal Microplate Reader, EL800, Bio-Tek Instrument, Inc., Winooski, VT).

To differentiate antibodies produced in response to immunization of avian HEV ORF2 protein from those against avian HEV infection, a peptide (named peptide 10) was synthesized and purified at Iowa State University Protein Facility and used as the differential antigen. Peptide 10 is located at N terminal antigenic domain V with the amino acid sequence of GRRGQRRRDNSAQWSTQQRPEGAV. The antigenic profiles on full-length avian HEV ORF2 (Fig. 3) showed that the antigenic domain V does not overlap with the C-terminal truncated avian HEV ORF2 protein used as the immunogen in this study. The differential ELISA was performed essentially as described previously [35], except that 200 ng of peptide 10 was coated in each well and sera were 1:100 diluted. Anti-avian HEV positive and negative chicken sera were used as controls.

2.8. Detection of serum lactate dehydrogenase (LDH)

It has been demonstrated that avian HEV infection results in the elevation of chicken serum LDH [2]. Cytotoxicity LDH detection kit (Roche Applied Science, IN) was used to measure the LDH levels in the chicken sera collected after virus challenge according to the manufacture’s instruction. Briefly, chicken sera were 1:10 diluted with freshly prepared 1%
bovine serum albumin (BSA) in 100mM PBS (pH 7.5). Chicken LDH standard (Sigma) was also serially diluted and used to establish a standard curve. Freshly mixed catalyst and dye solution (100 µl) provided in the kit were added into each well of 96-well ELISA plate, followed by adding 100 µl of diluted serum samples and chicken LDH in duplicates. The 1% BSA diluent was added and used as a background control. After incubation at room temperature for 20 min, the reaction was stopped by adding 1N HCl (50 µl/well) and measured with ELISA reader at 490 nm. The absolute LDH activity in chicken sera was inferred from the LDH standard curve.

2.9. Statistical analysis

Statistical analysis was performed as described previously [35]. P values of ≤0.05 were considered significant.

Results

3.1. Anti-avian HEV ORF2 response to immunization with recombinant ORF2 antigen

To generate a background binding value for the chicken serum samples, ELISA plates coated with avian HEV ORF2 antigen were reacted with 40 chicken serum samples negative for avian HEV. The average OD value was 0.1763 with a standard deviation (STDEV) of 0.0455. Therefore, an OD value of 0.358, which is 4 STDEV above the mean OD value of negative chicken sera, was used as the ELISA cut-off value. The anti-avian HEV ORF2 antibody titers in response to immunization with recombinant ORF2 antigen were defined as the last serum dilution that gives an OD value above 0.358. After 2nd immunization, the majority of the immunized chickens had a serum titer of approximately 1:10,000 (Fig. 1). At 1:1000 dilution, 6 chickens had OD values of >2.5, 9 chickens of 1.75-2.5 and 5 chickens of
1.0-1.75. The anti-avian HEV ORF2 antibody levels did not increase significantly in these chickens after the 3rd immunization (data not shown). As expected, the chickens that were mocked immunized with KLH precipitated in aluminum did not develop specific anti-avian HEV ORF2 antibodies (average OD value was 0.13).

3.2. Immunization of chickens with recombinant ORF2 prevents viremia and virus shedding in feces and bile

Serum, fecal and bile samples from all chickens were negative for avian HEV RNA at 0 WPC. The samples collected from 1 to 5 WPC from avian HEV ORF2 protein immunized chickens were all negative for avian HEV RNA (Table 1). In contrast, serum viral RNA was detected in mock-immunized chickens starting at 1 WPC and remained positive for at least another 4 weeks. Fecal viral RNA was detected in 14/20, 16/16, 11/12, 11/12 and 8/8 mock-immunized chickens at 1, 2, 3, 4 and 5 WPC, respectively. Avian HEV RNA was detected from bile samples in 4/4, 3/4, 1/4 and 2/8 mock immunized chickens at 1, 2, 4 and 5 WPC, respectively (Table 1). Sequencing results of the PCR products amplified from chicken #19 and #22 confirmed the recovered viruses were originated from the inoculum. These sequences were deposited within GeneBank with the accession numbers of DQ111980 and DQ111981.

3.3. Anti-avian HEV ORF2 responses after challenge with avian HEV

Serum samples collected from 16 chickens that were monitored consecutively from 1 to 5 WPC were tested to evaluate the kinetics of anti-avian HEV ORF2 antibody responses. In the 8 chickens immunized with avian HEV ORF2 protein, the anti-avian HEV ORF2 antibodies remained at high levels after the 2nd immunization but declined rapidly at 1 WPC
and remained at lower levels for at least another 4 weeks (Fig. 2). A transient increase of the anti-avian HEV ORF2 antibody level in chicken #37 occurred between 3 and 4 WPC and declined thereafter. The mock-immunized chickens were negative for anti-avian HEV ORF2 antibodies before challenge, and seroconverted to avian HEV antibodies in response to challenge at 3 WPC, and the antibody levels increased steadily for at least another 2 weeks.

3.4. Anti-peptide antibody responses after avian HEV challenge: differentiation of anti-avian HEV antibody responses induced by immunization from virus infection

Through the antigenic analysis of the avian HEV ORF2 protein, several potential antigenic domains were identified (Fig. 3) in addition to the previously identified four domains [34]. Domain V is most antigenic in the N terminus. Peptide 10 is within this domain and was used in the ELISA to detect and differentiate anti-avian HEV antibodies induced by the avian HEV challenge from the antibody response induced by immunization with truncated ORF2 protein. At 0 WPC (2 weeks after the third immunization) and 1 WPC, all 16 chickens had no detectable anti-peptide 10 antibodies (approximately 0.087 OD value) (Fig. 4). Anti-peptide 10 antibodies were not detected in chickens immunized with the avian HEV ORF2 recombinant protein from 1 to 5 WPC except for one chicken (#37) that had detectable anti-peptide 10 antibodies at 3 and 4 WPC with the OD values of 0.304 and 0.287, respectively ($P$ value of 0.01, 0.03, respectively). In contrast, anti-peptide 10 antibodies were detected from 8 mock-immunized chickens starting at or after 2 WPC and peaked at 5 WPC (OD values from 0.6 to 0.9).

3.5. Serum LDH

It has been shown that serum LDH enzyme was increased in chickens after
intravenous or oronasal inoculation with avian HEV [2]. In this study, serum samples collected from 16 chickens that were monitored consecutively from 1 to 5 WPC were also tested for LDH. The average serum LDH at 1 WPC from 8 mock-immunized chickens was 2.85 log (U/L) which was significantly higher than avian HEV ORF2 protein immunized chickens (2.3 log (U/L), $P$ value of 0.017). There was no significant difference for the levels of serum LDH at other WPC between the two groups of chickens (Fig. 5).

3.6. Gross hepatic lesions

Moderate to severe subcapsular hemorrhages were observed in the livers of 6 of 20 mock-immunized and subsequently challenged chickens during necropsies (Fig. 6). No liver lesion was observed in immunized and challenged chickens.

Discussion

Avian HEV infection is associated with a hepatitis disease, chicken HS syndrome. It has been demonstrated that avian HEV infection in chickens served as a valuable homologous small animal model for studying human HEV pathogenesis [2]. However, there is no report whether chickens can be used as model to study anti-HEV immune responses. Human HEV shares common antigenic epitopes in the ORF2 protein with avian HEV [34] and two common B cell epitopes were recently identified [35]. In this study, we demonstrated that chickens immunized with avian HEV ORF2 recombinant protein induced anti-ORF2 antibodies, and were protected from avian HEV infection and the development of hepatitis after challenge with avian HEV.

Previous studies showed that immunization with human HEV ORF2 protein expressed in SF9 cells protected monkeys against human HEV infection [21–24]. Recently,
human HEV ORF2 proteins have been expressed in bacteria and were found predominantly in a dimmer form [29,30]. Monkeys vaccinated with these proteins were completely protected against infection with a low dose virus, and partially protected with a high dose virus challenge [29,30]. Our results showed that a truncated avian HEV ORF2 recombinant protein in the monomer form expressed in bacteria [34,35] was highly immunogenic in chickens. Sixteen of 20 immunized chickens developed anti-avian HEV ORF2 antibodies with titer of approximately 1:10,000 after 2nd immunization (Fig. 1), although the antibody levels varied among chickens. The protective immunity in chickens immunized with avian HEV ORF2 protein was likely due to the induction of neutralizing antibodies against avian HEV ORF2 protein, which eliminates the challenge virus.

Anti-avian HEV ORF2 antibody levels in 8 chickens immunized with avian HEV ORF2 protein decreased dramatically at 1 WPC (Fig. 2), and this observation was confirmed by repeated testing of the samples. Although the reason for the rapid decrease of anti-avian HEV ORF2 antibodies after virus challenge is not known, we believe that the anti-avian HEV ORF2 antibodies induced by immunization were likely processed and degraded during the virus neutralization process against the challenge virus. It is known that the ingestion and degradation of antibody-virus complex by macrophages or natural killer cells contribute to the final clearance of circulating virus [38]. However, it cannot be ruled out that anti-avian HEV ORF2 antibodies were rapidly digested, which has been seen in other virus systems [39,40].

The transient increase of anti-avian HEV ORF2 antibodies in chickens #37 after avian HEV challenge suggested that some viruses may escape neutralization, and consequently
anti-avian HEV antibodies were induced as confirmed by the detection of anti-peptide 10 antibodies in this chicken (Fig. 4). As avian HEV RNA was not detected in serum, fecal or bile samples from this chicken, the replication level of the escaped viruses was limited. The reason for failing to completely neutralizing the challenge viruses in chicken #37 may be due to the lower level of neutralizing antibodies in this chicken as indicated by the relative low level of anti-avian HEV ORF2 antibodies induced by immunization (Figs. 1 and 2). Further study is warrant to examine the level of neutralizing antibodies in these chickens by *in vitro* neutralizing study using the purified chicken IgY.

Moderate to severe subcapsular lesions in the liver were observed in 6 of 20 mock-immunized chickens, and the mean serum LDH level was also transiently increased in these chickens (Figs. 5 and 6). The finding that only a portion of infected chickens developed gross liver pathological lesions was consistent with a previous report by Billam et al [2]. Chickens immunized with avian HEV ORF2 immunized and subsequently challenged with avian HEV did not have any observable gross liver lesions or increased serum LDH after virus challenge, indicating that immunization of chickens with avian HEV ORF2 recombinant protein prevented hepatitis caused by avian HEV infection.

The dominant neutralizing epitopes on the capsid protein of human HEV were located in the C-terminal regions of the ORF2 [16–19]. Our study also suggested that neutralizing epitopes of avian HEV are located on the C terminus of the ORF2 protein, since the recombinant ORF2 protein of avian HEV used in the study was the C-terminal part (aa 339–606) of the avian HEV ORF2 protein [35]. By using monoclonal antibody and series of recombinant avian HEV ORF2 proteins, two different neutralization epitopes (aa 138–175
and aa 175–232) were identified (our unpublished results), further indicating that the protective immunity of avian ORF2 protein was directly related to the induction of neutralization antibodies.

Acknowledgements

This work is supported in part by a grant (to X.-J. M.) from the National Institutes of Health (AI 50611).

References


**Table 1.** Detection of avian HEV RNA in sera, fecal swabs and bile samples from chickens challenged with avian HEV virus.

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<tr>
<th>Chicken Group</th>
<th>No. of positive sera (fecal swabs) / total no. tested at indicated WPC</th>
<th>No. of positive bile samples / total no. tested at indicated WPC</th>
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<td>0(0)/20</td>
</tr>
<tr>
<td>Group 2&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>: chickens were immunized with avian HEV ORF2 recombinant protein.

<sup>b</sup>: chickens were mock immunized.

<sup>c</sup>: not done.
**Figure Legends**

**Fig. 1.** Anti-avian HEV ORF2 antibody titration in chickens immunized with the recombinant avian HEV ORF2 protein. Serum samples collected from 20 chickens at 2 weeks after 2nd immunization with the recombinant avian HEV ORF2 protein were titrated for level of anti-avian HEV ORF2 antibodies. Avian HEV ORF2 protein was used as the antigen at 100 ng/ml on the solid phase of ELISA plates. Each point represents the mean values (±S.E.M.) of the OD 490 nm obtained from the 20 chickens.

**Fig. 2.** Kinetics of anti-avian HEV ORF2 antibody responses in immunized chickens before and after challenge with avian HEV. Sera from 16 chickens were diluted at 1:1000 and tested for the presence of anti-avian HEV ORF2 antibodies by ELISA. Each bar represents the mean value (±S.E.M.) of OD 490 nm obtained from 16 chickens. Chickens # 17 to #24 were mock immunized. Chickens # 37 to #44 were immunized with the recombinant avian HEV ORF2 protein.

**Fig. 3.** Hydrophilicity, antigenicity and surface probability plots of the full-length avian HEV ORF2 protein. Antigen profiles on the full-length avian HEV ORF2 protein were analyzed by the Protean program (DNA STAR Inc., Madison, WI). Shaded area indicates the C-terminal 268 aa residues of avian HEV ORF2 protein containing 4 predicted putative antigenic domains [34]. Antigenic domain V was identified as the most antigenic domain through the combination of hydrophilicity, antigenicity and surface probability of the amino acids (dot boxed area). Peptide 10 was truncated from this domain. Sequence of this peptide was given in the text.

**Fig. 4.** Kinetics of anti-peptide 10 antibodies in immunized chickens after challenge with
avian HEV. Serum samples (diluted 1:100) from 16 chickens were tested for the presence of anti-peptide 10 antibodies. Each point represents the mean values (±S.E.M.) of OD 490 nm obtained from 16 chickens. Chickens #17 to #24 were mock immunized. Chickens #37 to #44 were immunized with the recombinant avian HEV ORF2 protein.

Fig. 5. Serum levels of LDH in chicken sera after avian HEV challenge. The log mean LDH values at each WPC were generated from 8 mock-immunized and 8 avian HEV ORF2 protein immunized chickens. *, Statistical significance ($P \leq 0.05$).

Fig. 6. Gross hepatic lesions in chickens challenged with avian HEV. Moderate (upper) to severe (lower) subcapsular liver hemorrhages (arrows) were found in 6 of 20 mock-immunized chickens after virus challenge as indicated. Livers with moderate gross hepatic lesions were observed in chickens necropsied at 1 and 2 WPC, whereas livers with severe lesions were seen in chickens necropsied at 4 and 5 WPC.
Protection of chickens against avian HEV infection by immunization of recombinant avian HEV ORF2 protein

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Fig. 2.
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Fig. 5.
Protection of chickens against avian HEV infection by immunization of recombinant avian HEV ORF2 protein

H. Guo, E. M. Zhou, Z. F. Sun, X. J. Meng

Fig. 6.
CHAPTER 5. IDENTIFICATION OF TWO NEUTRALIZATION EPITOPEs ON 
THE CAPSID PROTEIN OF AN AVIAN STRAIN OF HEPATITIS E VIRUS BY 
EPITOPE MAPPING

A paper submitted to Journal of Virology

H. Guo¹, E. Zhou², F. F. Huang³, Z. F. Sun³, X. J. Meng³

Abstract

Avian hepatitis E virus (avian HEV), an emerging virus in chickens, is genetically and 
antigenically related to human hepatitis E virus, the causative agent of hepatitis E. To 
identify the neutralizing epitopes on the capsid protein of avian HEV, four monoclonal 
antibodies (Mabs) with IgM isotype (7B2, 1E11, 10A2, 5G10) against the recombinant avian 
HEV capsid protein were generated. 7B2, 1E11, 10A2 blocked each other for binding to 
avian HEV ORF2 protein in a competition ELISA, whereas 5G10 did not block other Mabs, 
suggesting that 7B2, 1E11, 10A2 recognize the same or overlapping epitopes and 5G10 
recognizes a different one. The epitopes recognized by 7B2, 1E11, 10A2 were further 
mapped between aa residues 175 and 232, and the epitope recognized by 5G10 was between 
aa residues 138 and 175 as determined by Western blot with the use of 5 avian HEV ORF2 
recombinant proteins. 1E11, 10A2 and 5G10 were shown to bind to bona fide avian HEV.

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particles *in vitro*, although only 5G10 reacted to viral antigens in transfected LMH chicken liver cells. To assess the neutralizing activities of the Mabs, avian HEV virus was mixed with each Mab or normal mouse IgM and incubated *in vitro* before inoculation into SPF chickens. Chickens inoculated with mixtures of avian HEV and 1E11, 10A2 or 5G10 had viremia and fecal virus shedding delayed at least two weeks, compared to chickens inoculated with a mixture of avian HEV and 7B2 or normal mouse IgM, suggesting that 1E11, 10A2 and 5G10 can partially neutralize avian HEV.

**Running title**: Two neutralization epitopes on avian HEV capsid protein.

**Introduction**

Hepatitis E virus (HEV) is a single-stranded, positive-sense RNA virus with an approximate 7.2 kb genome packaged within a non-enveloped capsid (9, 18). The HEV genome contains three open reading frames (ORFs 1, 2 and 3), which encode, the non-structural viral proteins, capsid protein, and a small protein that may be involved in modulating cell signaling (9, 40), respectively. HEV infection causes frequent endemic and rare epidemic outbreaks of acute hepatitis E in many developing countries. The prevalence of IgG HEV antibody was reported in approximately 1–20 % of the population from industrialized countries (28, 29). Recently, increasing numbers of cases of acute hepatitis E not associated with travel to endemic regions have been reported in numerous industrialized countries, including the United States (1, 23, 26, 34).

The first animal strain of HEV, swine HEV was isolated from pigs in the United States in 1997 (24). Recently, avian HEV, was identified in the United States from chickens with hepatitis-splenomegaly (HS) syndrome (14). Pathological lesions characteristic of HS
syndrome have been reproduced in specific-pathogen-free (SPF) chickens experimentally infected with avian HEV (6). Avian HEV has been classified as a putative 5\textsuperscript{th} genotype within the genus *Hepevirus* (11), which also includes human and swine HEVs. Although the genome of avian HEV is approximately 600 bp shorter than that of swine and human HEVs (16), it shares 50-60% nucleotide sequence identity, common antigenic epitopes and significant other functional features with the mammalian HEVs (12, 13, 16).

The anti-HEV humoral immune response is sufficient to confer protective immunity (7, 41). The ORF2 capsid protein is the most immunogenic and responsible for the induction of protective humoral immune responses (22, 31, 32, 43). As a result, currently, the ORF2 capsid protein is the target of vaccine designs and the recombinant proteins expressed either in *Escherichia coli* or insect cells have been used for evaluation of vaccine efficacy (10, 17, 21, 30). Broadly reactive anti-HEV neutralizing antibodies are induced in rhesus monkeys by vaccination with genotype 1 HEV recombinant capsid protein, which protected rhesus monkeys from challenge with viruses of the other three mammalian genotypes (30). Convalescent sera from animals infected with one of the four mammalian genotypes can neutralize all other genotypes (8). Furthermore, a major neutralizing epitope was identified on the HEV Sar-55 ORF2 capsid protein (36, 37) and Mabs raised against this epitope neutralized two mammalian HEV genotypes (8).

Four major antigenic domains at C terminal 268 amino acid residues of avian HEV capsid protein were predicted (13). Domain I is the most conserved among avian, human and swine HEVs, whereas domain IV is more antigenic in avian HEV than in swine and human HEVs. By using 9 synthetic peptides derived from these four antigenic domains, we
previously demonstrated that B cell epitopes in the antigenic domain I are shared among avian, human and swine HEV capsid proteins, and epitopes in domain IV are shared between avian and human HEVs, and one epitope in domain III is unique to avian HEV (12). However, our unpublished data showed that none of the rabbit antibodies raised against these peptides could neutralize low infectious doses of avian HEV, suggesting that the potential neutralizing epitopes are either located in other region(s) or they are non-lineal. To identify the potential neutralizing epitopes, we first generated Mabs against avian HEV ORF2 capsid protein and their abilities to neutralize avian HEV were subsequently investigated in chickens. In addition, series of truncated avian ORF2 recombinant proteins were expressed and used to map the epitopes recognized by these Mabs.

Materials and Methods

**Antigen preparation.** The recombinant pRSET-C plasmid harboring the truncated ORF2 capsid gene of avian HEV was produced as described previously (13). Recombinant avian HEV ORF2 capsid protein was expressed as inclusion body with an estimated molecular weight of 32 kDa and purified according to the methods described elsewhere (12). Ten mg of the purified protein was precipitated in aluminum as previously described (35).

**Immunization.** Four BALB/c mice were injected intraperitoneally with 100µg of prepared avian HEV ORF2 capsid protein followed by two booster doses at two-week interval. Mice were bled before each injection and two weeks after the 2\textsuperscript{nd} and 3\textsuperscript{rd} injection.

**Hybridoma production.** Two weeks after the 3\textsuperscript{rd} immunization, mice showing highest antibody titers were selected as the spleen cell donors. Four days prior to the fusion, the donor mice were given a final boost injection of 100 µg of the antigen. Hybridomas were
produced by the fusion of spleen cells with SP2/0 murine myeloma cells by standard PEG-mediated fusion methods.

**Indirect ELISAs.** Hybridomas were screened for secreting Mabs with an indirect ELISA as described previously (12) with the following modifications. Briefly, ELISA plates were coated with avian HEV ORF2 capsid protein overnight at 4°C. After washing and blocking the plates, 100μl hybridoma supernatants were added to each well in duplicates and incubated for 1 hr. HRP-conjugated goat anti-mouse IgG + IgM(H+L) (Jackson ImmunoResearch) was added as detecting antibodies. *O*-phenylenediamine dihydrochloride (OPD) (Sigma) was used as the substrate. Supernatants from hybridoma cells with OD values of 3 times higher than that from SP2/0 cells were considered positive and the corresponding hybridoma cells were subjected to two more rounds of cloning and selection in order to establish the stability and specificity. A total of four Mabs was obtained with the isotypes of IgM with a κ-light chain as determined by a mouse monoclonal antibody isotyping kit (Bio-Rad, CA).

To test the specificity of Mabs, four other recombinant proteins of human HEV Sar-55 ORF2 (33), swine HEV ORF2 (25), porcine reproductive and respiratory syndrome virus (PRRSV) nucleocapsid (N) and porcine circovirus (PCV) ORF2 proteins (27) and nine synthetic peptides truncated from avian ORF2 antigenic domains (12) were coated in ELISA plates at 1μg/ml overnight at 4°C. After blocking and washing the plates, Mabs at various dilutions were added into each well and incubated for 1 hour. HRP-goat anti-mouse IgM, μ chain specific (Jackson ImmunoResearch) was used as the secondary antibody.
**Flow cytometry analysis.** The surface IgM profiles of the hybridomas were analyzed by flow cytometry on a Beckton-Dickinson FACScan (Beckton-Dickinson, CA). Hybridoma cells (1 x 10^6) were stained with FITC-AffiniPure Monovalent Fab Fragment Goat Anti-Mouse IgM, μ chain specific (Jackson ImmunoResearch) in accordance with a standard protocol provided by Flow Cytometry Facility at Iowa State University. Normalized mean fluorescence intensities (MFI) were calculated using CellQuest software (Beckton-Dickinson, CA).

**Competitive ELISA.** The spatial relationships of epitopes recognized by each of the Mabs were evaluated by a pair-wise competitive ELISA essentially as described elsewhere (44) except that the avian HEV ORF2 capsid protein was used as the coating antigen and the residual binding of biotinylated Mabs was detected using HRP-conjugated streptavidin (Jackson ImmunoResearch, PA) and TMB substrate system (KPL, MD). Biotinylated Mabs (B-Mabs) were used at a dilution that gives an ELISA result of approximately 1.0 optical density (OD) (in the linear portion of their binding curve to avian HEV ORF2 antigen). Maximum binding (no inhibition) was determined when B-Mabs were added without competitors. The percentage of inhibition (PI) was calculated using the formula: $100 \times [1 - (\text{OD}_{450\text{nm of B-Mab and Mab}}) / (\text{OD}_{450\text{nm of B-Mab}})]$. A B-Mab was considered inhibited if its binding was decreased by 40% or more.

**Purification and biotinylation of Mabs.** Mabs were purified from culture supernatants using anti-mouse IgM (μ-chain specific) agarose affinity column (Sigma). The purified Mabs were dialyzed against 0.01M PBS buffer (pH 7.2) and subsequently concentrated to 2 mg/ml using Minicon B15 concentrator (Millipore, MA) for biotinylation.
using EZ-link-Sulfo-NHS (N-hydroxysuccinimide)-LC-Biotin (Pierce, Rockford, IL) in accordance with the manufacturer’s protocol.

**Indirect immunofluorescence assay (IFA).** An IFA was used to further test the reactivity of Mabs with avian HEV ORF2 antigen. Briefly, Capped RNA transcripts from a full-length infectious cDNA clone of avian HEV, pT7-aHEV-5, were used to transfect LMH chicken liver cells, which supports avian HEV replication (15). Mabs were used at 10µg/ml and fluorescein-labeled anti-mouse IgM (KPL) was used at 1:50 dilution. The remaining procedures were performed as previously reported (15).

**Binding of Mabs to avian HEV virus.** A modified immuno-capture RT-PCR method (20) was used to assess the virus binding ability of the Mabs. Briefly, 100ng, 200ng and 400ng of Mabs or normal mouse IgM (κ-light chain) (Sigma) were coated on the solid-phase of ELISA plates (Nunc™, USA). Serum samples containing avian HEV from viremic chickens were added at 100µl/well and incubated at room temperature for 1 hour and then at 4°C for 12 hours. After 6 times washing, 100µl of viral RNA extraction buffer from RNAgents® Total RNA Isolation System (Promega) was directly added into each well. The RNA was then extracted and reverse transcribed into cDNA with AMV reverse transcriptase (Promega). A nested RT-PCR (39) was used to detect avian HEV viral helicase gene. Final PCR products were subjected to electrophoresis on a 1.2% agarose gel containing ethidium bromide and the second round PCR products of 221 bp was confirmed by DNA sequencing. Experiments were repeated two more times.
**Neutralization assay.** Fifteen anti-avian HEV negative chickens were divided into 5 groups with 3 chickens each group. From the result of virus capturing assay described above, we estimated that 1mg of Mabs of 1E11, 10A2 and 5G10 could bind at least 5000 genome equivalent (GE) of avian HEV virus. Thus, 3000 GE of avian HEV virus was incubated with 3mg of each of the four Mabs or normal mouse IgM. The mixtures were incubated at room temperature for 1 hour and at 4°C overnight, and each mixture was subsequently injected equally into each of the three chickens via intravenous route. Sera and fecal swabs were collected before inoculation and weekly thereafter for 5 weeks. Viremia and fecal virus shedding were detected by a sensitive nested RT-PCR as previously described (39).

**Cloning and expression of avian HEV ORF2 fragment proteins.** As anti-HEV neutralization epitopes are most likely located in the C terminal part of the ORF2 capsid protein (37, 42), in order to further map the epitopes recognized by anti-avian HEV ORF2 Mabs, four C-terminally truncated avian ORF2 fragments, designated avian ORF2-1 to avian ORF2-4 were constructed (Fig. 4) with recombinant plasmids containing 804 bp avian HEV ORF2 gene (13) as the template. To amplify these 4 fragments, four downstream primers were designed using Oligo 6.0 software (MBI, Cascade, CO). EcoR I restriction site was engineered into the 5’ end of these primers to facilitate subsequent cloning steps (Table 4). The forward primer containing BamH I site used for PCR amplification of avian HEV ORF2 (13) was suitable for all the amplification of all four fragments. Proofreading *Pfu* DNA polymerase (Stratagene) was used for all PCR amplifications. The PCR conditions were performed as following: 1 cycle at 95°C for 5 minute; 35 cycles at 95°C for 45 sec, 51°C for 45 sec and 72°C for 45 sec; 1 cycle at 72°C for 10 minutes. PCR products were digested and
purified with Wizard® SV Gel and PCR Clean-Up System (Promega). Purified DNA fragments were cloned into linearized pRSET-C expression vector (Invitrogen), which encodes a histidine tag and an Xpress™ epitope at the N terminus of the multiple cloning sites. The subsequent expression of these fragments was performed essentially as previously described (13), except for avian HEV ORF2-1. For the expression of avian HEV ORF2-1, the incubation was performed with a shaking speed of 225 rpm at 25°C for 6 hours after adding 1 mM IPTG. The fusion proteins were partially purified from 100 ml of IPTG-induced bacterial cultures with the BugBuster Protein Extraction Reagent (Novagen). Expressions of the four truncated avian HEV ORF2 proteins were analyzed by Western blot (13) using 1:5000 diluted HRP-conjugated anti-Xpress epitope Mabs (Invitrogen) or 1:1000 diluted polyclonal anti-avian HEV chicken sera.

**Mapping of neutralizing epitopes on avian HEV capsid protein and comparison with that of Sar-55 human HEV.** The epitopes recognized by the Mabs were mapped by Western blot. Each of the partially purified truncated avian HEV ORF2 proteins was separated by 15% SDS–PAGE in non-reducing or reducing conditions and electrophoretically transferred onto a 0.2μM polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were blocked with 0.01M PBST containing 2.5% dry milk overnight with slow shaking and then incubated for 1 hour at room temperature with each of the Mabs at 4μg/ml. Normal mice sera (1:100) and mouse anti-avian ORF2 sera (1:1000) were used as negative and positive controls, respectively. After washing the membranes, HRP-goat anti-mouse IgM (μ chain specific) (1:2000) was added and incubated for 1 hour. The immunocomplexes were
detected using 3, 3'-Diaminobenzidine (DAB) Enhanced Liquid Substrate System tetrahydrochloride (Sigma).

The identified neutralizing epitope positions on avian HEV ORF2 protein were compared to that identified on human HEV Sar-55 ORF2 protein (36, 37, 45). The 268 aa avian HEV ORF2 protein and the corresponding 267 aa Sar-55 ORF2 protein (13) were aligned with MegAlign program (DNAstar package, DNA STAR Inc., Madison, WI) by ClustalW alignment with the selection of Fast-Approx and BLOSUM 30 matrix.

Results

Production and characterization of Mabs. Four hybridoma clones secreting Mabs designated 7B2, 1E11, 10A2, and 5G10 (all IgM isotypes with κ-light chain) against avian HEV ORF2 capsid protein were obtained from four different parental fusion clones, and confirmed by detection of the hybridoma surface IgM profiles (MFI for 7B2, 1E11, 10A2, and 5G10 is 107.8, 157.9, 308.6 and 354.4, respectively) (Table 1). Since IgM antibodies have poly-reactivity (3, 19), the specificity of these Mabs was first tested with different antigens. As seen in Fig.1, all Mabs reacted specifically with avian HEV ORF2 capsid protein but not with avian HEV ORF2 peptides, the ORF2 capsid proteins of genotype 1 human and genotype 3 swine HEVs, PCV capsid protein, or PRRSV N protein. At 1µg, the OD490nm values reacting with avian HEV capsid protein were 2.5, 2.3, 2.2 and 1.1 for 5G10, 10A2, 1E11 and 7B2, respectively. The 7B2 had the lowest binding affinity whereas 5G10 had the highest. 1E11, 10A2 had similar binding affinity to 5G10.
The binding of B-7B2 to avian HEV ORF2 protein was inhibited by homologous 7B2 at 84.8%. Complete inhibition was obtained for heterologous 1E11 and 10A2 with the percentage of inhibition at 95.2 and 96.8, respectively (Table 2). The binding of B-1E11 and B-10A2 to avian HEV ORF2 protein was completely inhibited by the homologous Mabs, and inhibited each other (93%-98% inhibition). In contrast, 7B2 can only partially inhibit the binding of B-1E11 and B-10A2 to the avian HEV ORF2 protein at 54.4% and 63.1%, respectively. These results suggested that 7B2, 1E11 and 10A2 may share the same or overlapping epitopes. There was no significant inhibition (<19%) between 5G10 and the other three Mabs, indicating that 5G10 likely recognizes a different epitope.

To determine whether Mabs could recognize the native avian HEV ORF2 capsid protein on viral particles, LMH chicken liver cells were transfected with capped RNA transcripts from a full-length infectious clone of avian HEV (15). The reactivity of Mabs with the native avian HEV ORF2 protein in transfected LMH cells was examined by IFA. The positive control polyclonal anti-avian HEV serum reacted with avian HEV ORF2 proteins on the transfected cells (Fig. 2A), while negative chicken sera did not react with the transfected cells (Fig. 2C). At the concentration of 10µg/ml, only 5G10 showed reproducible binding to the native avian HEV ORF2 capsid protein (Fig. 2B). None of the other three Mabs reacted with the transfected cells (data not shown).

**Mabs reacted with avian HEV particles.** To determine whether Mabs could capture circulating avian HEV particles, a sensitive nested RT-PCR (39) was used to detect the presence of avian HEV captured by the Mabs. As shown in Fig.3, 1E11, 10A2 and 5G10, when coated at 200ng or 400ng per well, captured avian HEV. At 100ng/well, 5G10 was
also able to capture avian HEV. Neither 7B2 nor control mouse IgM could capture the virus with the amounts used.

**Neutralization of avian HEV by Mabs.** Since there is no cell culture available to support the replication of avian HEV, an animal-based neutralization assay was used in this study to examine the ability of Mabs to neutralize avian HEV as previously established (36). Chickens that were inoculated with avian HEV incubated with either normal mouse IgM or 7B2 became infected one week after inoculation, as evidenced by fecal virus shedding and viremia (Table 3). In contrast, chickens inoculated with avian HEV incubated with 1E11, 10A2 and 5G10 did not have detectable avian HEV RNA in sera or feces until 3 weeks post-inoculation. These results suggested 1E11, 10A2 and 5G10 could at least partially neutralize avian HEV and the infection caused by non-neutralized virus or escaped virus was significantly delayed.

**Expression and purification of avian HEV ORF2 fragments.** Avian HEV ORF2-1, 2, 3, 4 fragments were amplified by RT-PCR (Fig.5A), and subsequently cloned into pRSET-C expression vector. The recombinant clones were confirmed by DNA sequencing. After 3 hours incubation in a shaker with 250 rpm at 37°C, the ORF2-2, 3, 4 proteins with expected sizes of approximately 23, 18, 16 kDa, respectively, were expressed and partially purified (Fig. 5B). Two proteins with the sizes of approximately 28kDa and 16.5kDa were obtained when bacterial cells transformed with avian HEV ORF2-1 plasmids were induced with IPTG and cultured at 25°C in a shaker at 225 rpm for 6 hours (Fig. 5B). All fusion proteins were expressed predominantly as inclusion bodies in bacterial cells. Western blot analysis using a monoclonal antibody against the Xpress epitope confirmed the expression of
these recombinant ORF2 proteins (Fig. 5C). The expressed proteins were also recognized by anti-avian HEV positive chicken sera in Western blot (Fig. 5D).

**Epitope mapping.** To map the epitopes recognized by the Mabs, the purified avian HEV ORF2 fragment proteins were probed with each Mab in Western blot analyses. As shown in Fig.6, all Mabs recognized ORF2 and ORF2-1 proteins, but not the other proteins, suggesting that at least one epitope is located between aa residues 175 and 232 (Fig.4 and Fig.6). In addition to the ORF2 and ORF2-1 proteins, 5G10 also recognized the ORF2-2 protein, suggesting that the recognized epitope by 5G10 is located between aa residues 138 and 175. The reaction pattern for each MAb was same when avian HEV ORF2 fragment protein samples were loaded in reducing sample buffer (data not shown). These results were in agreement with the competitive ELISA results (Table 2), which implied that 7B2, 1E11, 10A2 and 5G10 recognized two different epitopes.

The avian HEV ORF2 protein has similar antigenic profiles to that of genotype 1 human HEV (Sar-55 strain), and the relative positions of the predicted four antigenic domains on ORF2 protein are similar between avian HEV and Sar-55 HEV (13). It has been reported that the neutralizing epitope on Sar-55 ORF2 protein was between aa 459 and 607 (8, 45) or aa 66 to aa 214 (Fig.7), and it covered all aa residues in domain II and most of aa between domains II and III (Fig. 7). Although this neutralizing epitope spans a 148 aa region in length, it is the shortest that can be recognized by the corresponding neutralizing Mabs (8, 45). In this study, the neutralizing epitope defined by 5G10 was between aa residues 138 and 175 and covered most of aa residues in domain II and part of aa between domains II and III (Fig. 7). The neutralizing epitope recognized by 7B2, 1E11, 10A2 was between aa residues
175 and 232 and covered all the aa of domain III and part of aa between domains II and III (Fig. 7). Therefore, the relative positions of the two neutralizing epitopes on avian HEV capsid protein identified in this study are similar to the mapped neutralizing epitope on Sar-55 ORF2 protein (Fig. 7).

Discussion

Avian HEV has been classified in the same genus *Hepevirus* as the mammalian HEVs (11). There exist similar antigenic profiles between avian HEV and human HEV ORF2 capsid proteins (13). Numerous studies have demonstrated that immunization of monkeys with recombinant ORF2 protein of human HEV expressed in SF9 cells or bacterial cells confer protection against HEV infection by inducing strong antibody responses (10, 17, 21, 22, 31, 43). The Sar-55 HEV derived recombinant ORF2 protein has been used as candidate vaccine in phase II/III field trials in Nepal (30). Our recent data showed that immunization of chickens with bacterially expressed avian HEV ORF2 protein also induce protective antibody response (Guo et al., *Vaccine*. Submitted). The delineation of the neutralizing epitopes on avian HEV ORF2 capsid protein provided direct evidence that protection against avian HEV infection by immunization with avian HEV ORF2 protein was likely due to the induction of neutralizing antibodies.

Four anti-avian HEV ORF2 Mabs of IgM class were produced. It is unclear why only IgM isotypes were produced in this study, however inefficient switching was not likely since after the 2nd immunization, mouse anti-avian HEV ORF2 serum IgG titer reached about 1:100,000 in all immunized mice (data not shown). The competitive ELISA results indicated that 7B2, 1E11 and 10A2 recognize the same or overlapping epitopes and 5G10 recognizes a
different one. These results were further confirmed by the epitope mapping study. It is not surprising that 7B2 only partially inhibited the binding of B-1E11 and B-10A2 to the avian HEV ORF2 protein, since the binding affinity of 7B2 is almost two-fold lower than 1E11 and 10A2 (Fig. 1).

Two antigenic epitopes common to avian and human HEVs mapped previously by using synthetic peptides (12) were not recognized by these Mabs (Fig.1). Similarly, none of these Mabs recognized swine or human HEV recombinant ORF2 proteins, indicating that the corresponding epitopes recognized by these Mabs were unique to avian HEV ORF2 protein. If these epitopes are predominant neutralizing epitopes, cross-species neutralizing epitopes on HEV ORF2 proteins may not exist.

The IFA results using transfected LMH cells suggested the epitope recognized by 5G10 was intact whereas the epitopes recognized by 7B2, 1E11 and 10A2 were either damaged or not exposed on native avian HEV ORF2 proteins. It is important to point out that, upon transfection into the LMH cells, the RNA transcripts of the avian HEV infectious clone only have a limited level of replication and the replicating virus does not spread from cell to cell (15), and thus the IFA is much less sensitive to assess the binding between Mabs and virus capsid protein. The fact that 1E11 and 10A2 can capture circulating avian HEV particles suggested that the corresponding epitopes on viral ORF2 protein are exposed and recognized. The viral capturing ability of these Mabs was specific and affected by their affinity, as normal mouse IgM and lower affinity 7B2 did not capture any detectable avian HEV, whereas the higher affinity Mab 5G10 could capture virus in a relatively low amount. The demonstrated virus capturing ability of Mabs 1E11, 10A2 and 5G10 suggested that these
Mabs can neutralize virus. It has been reported that two anti-Sar-55 HEV chimpanzee Mabs can capture and neutralize Sar-55 HEV, whereas other Mabs without virus capturing ability cannot neutralize the virus (37).

It is not surprising that even at the same used amount as 1E11 and 10A2, 7B2 could not neutralize avian HEV due to its low affinity and virus capturing ability. The results that 1E11, 10A2 and 5G10 can only partially neutralize avian HEV proved that high levels of Mabs or convalescent anti-HEV sera are needed for complete virus neutralization (8, 42), and this could explain why some infected birds with low level of avian HEV antibody response are still viremic and shed virus in feces (6, 15, 39).

In a typical induction (1mM IPTG) and incubation condition (37°C and 250 rpm), the predicted 28 kDa avian HEV ORF2-1 fragment protein was not produced, but a significant amount of 16.5 kDa protein (data not shown). After excluding the possibilities of potential contamination with avian HEV ORF2-4 plasmid, which expresses an estimated 16 kDa protein, and the potential premature stop of avian HEV ORF2-1 gene introduced during RT-PCR and cloning steps, we speculate that the predicted 28 kDa avian HEV ORF2-1 fragment protein may be quickly degraded. To optimize this protein’s expression, we performed the induction and incubation conditions for ORF2-1 fragment protein at lower temperature and shaking speed (25°C and 225 rpm), since inclusion body proteins are easier to be improperly folded or irredeemably damaged and thus become target for degradation when bacterial cells grow at high speed and high temperature (5, 38). The use of low temperatures also has the combined advantages of slowing down transcription and translation rates and of reducing the strength of hydrophobic interactions that contribute to protein misfolding (4). Under the
modified expression condition for ORF2-1 fragment protein, the predicted 28 kDa protein was produced (Fig. 5B), indicating that the modified expression conditions significantly reduce the degradation rate of the expected 28 kDa protein. The specific truncation of avian HEV ORF2-1 fragment protein might produce a favorable conformation targeted for degradation, which did not occur in other ORF2 fragments.

The epitope defined by 5G10 was between aa residues 138 and 175. However, since the peptide 2 derived from the entire domain II (aa 123–154) could not react with 5G10, the actual epitope recognized by 5G10 could be between aa 154 and 175 if it is linear. The epitope recognized by 7B2, 1E11 and 10A2 was between aa 175 and 232. Similarly, since the peptide 3 representing entire domain III (aa 217–aa 227) failed to react with 7B2, 1E11 and 10A2 (Fig. 1, 7), amino acids of peptide 3 are not critical components of the epitope recognized by these three Mabs or the recognized epitope is probably conformational.

The reported human HEV Sar-55 neutralizing epitope is the only major protective epitope mapped on human HEV and it is the essential component of human HEV recombinant ORF2 protein vaccine (30, 37). Since the two neutralizing epitopes identified on avian HEV ORF2 protein in this study are nearly within the corresponding position of the Sar-55 neutralizing epitope, these avian HEV neutralization epitopes could also be the major protective epitopes on avian HEV ORF2 protein. However, neither of the two epitopes was conserved among avian, human and swine HEVs, as the corresponding Mabs failed to react with Sar-55 or swine HEV ORF2 antigens. This is not surprising, since the aa sequence identity of the epitope is only approximately 16% among avian, Sar-55 and swine HEVs. Two different epitopes on ORF2 protein of a genotype I HEV (strain XM) were identified by
Mabs 8C11 and 8H3 as protective neutralizing epitopes, as rhesus monkeys inoculated with the virus pretreated with these two Mabs had delayed onset of fecal virus shedding (2, 42). Although the detailed positions of these two epitopes were not mapped, they are within the same region of that of Sar-55 HEV, which is also a genotype 1 HEV (11). This suggests that there are likely more than one protective neutralizing epitopes for human HEV, which is in agreement with our findings in this study on avian HEV ORF2.

The avian HEV ORF2 protein used for immunization for the production of Mabs was purified from denatured form (12), thus the Mabs likely recognize linear epitopes, which was further confirmed by Western blot results. However, the originally-thought linear protective epitope on Sar-55 HEV ORF2 protein (36, 37, 45) has now been considered as conformational since it is very stable and resistant to heat treatment and reducing agents (36, 37). Thus, whether the neutralizing epitopes identified on the avian HEV ORF2 are conformational or linear warrants further investigation.

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References


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FIGURE LEGENDS

**Fig. 1.** Reactivity of anti-avian HEV ORF2 Mabs with different antigens. ELISA plates coated with different antigens were incubated with indicated concentrations of Mabs and the bound Mabs were detected with HRP-conjugated anti-mouse IgM, μ chain specific. Each point represents the mean value (±S.E.M.) of the OD_{490nm} obtained from duplicate ELISA results.

**Fig. 2.** Immunofluorescent staining of LMH chicken liver cells transfected with capped RNA transcripts from avian HEV cDNA infectious clone, pT7-aHEV-5 (15). (A) transfected cells stained with polyclonal anti-avian HEV chicken serum. (B) transfected cells stained with 5G10. (C) transfected cells stained with negative chicken serum.

**Fig. 3.** Capture of circulating avian HEV particles with Mabs. Nested RT-PCR was used to detect bound virus as described (39). Lane M: 1 kb ladder; lanes 1-3: coated with 100ng, 200ng and 400ng per well of 5G10, respectively; lanes 4-6: coated with 100ng, 400ng and 200ng per well of 10A2, respectively; lanes 7-9: coated with 100ng, 400ng and 200ng per well of 1E11, respectively; lanes 10-12: coated with 100ng, 200ng and 400ng per well of 7B2, respectively; lanes 13-15: coated with 100ng, 200ng and 400ng per well of normal mouse IgM, respectively.

**Fig. 4.** Schematic diagram of C-terminal-truncated avian HEV ORF2 protein fragments. Recombinant plasmid containing avian HEV ORF2 was used as the template for the construction of the four C terminally truncated ORF2 fragments as described in the text.

**Fig. 5.** Expression of avian HEV ORF2 protein fragments. (A) PCR amplification of avian HEV ORF2 fragments. M: DNA marker; lane 1 to 4: avian HEV ORF2-4, 3, 2, 1, with
expected sizes of 330, 414, 525, 696 bp, respectively. (B) SDS-PAGE of partially purified avian HEV ORF2 fragment proteins. M: Protein marker; lanes 1 to 4: avian HEV ORF2-4, 3, 2, 1, with predicted sizes of 16, 18, 23, 28 kDa, respectively; lane 5: 32 kDa avian HEV ORF2 protein (13). Avian HEV ORF2-1 had two major forms: a predicted 28 kDa, and a partially degraded 16.5 kDa. (C) Western blot of expressed products by using HRP-conjugated anti-Xpress epitope Mab. (D) Western blot analysis of the expressed proteins with ant-avian HEV chicken serum. Lanes in (C) and (D) are the same as in (B).

Fig. 6. Epitope mapping on avian HEV ORF2 capsid protein by Western Blot. Partially purified avian HEV ORF2 fragment proteins were separated on SDS-PAGE in non-reducing conditions and transferred to PVDF membranes and reacted with (A): 7B2; (B): 1E11; (C) 10A2; (D) 5G10. M: Protein marker; lanes 1 to 5: Mabs reacted with avian HEV ORF2 and avian HEV ORF2-1, 2, 3, 4, respectively.

Fig. 7. Comparison of neutralization epitopes on avian HEV and human HEV Sar-55 ORF2 proteins. Amino acid sequences of avian HEV ORF2 protein and its corresponding region of Sar-55 HEV were aligned. Dots represent identical residues. Dashes represent deletions. Two neutralizing epitopes on avian HEV ORF2 protein were boxed with solid and dot lines. The reported neutralizing epitope on Sar-55 HEV ORF2 protein (45) was underlined. The predicted four antigenic domains (I, II, III, IV) on avian HEV ORF2 capsid protein (13) were also indicated.
Table 1. Characterization of hybridomas

<table>
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<tr>
<th>Hybridomas</th>
<th>Isotype</th>
<th>MFI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Affinity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>IFA&lt;sup&gt;c&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>7B2</td>
<td>IgM/κ</td>
<td>107.8</td>
<td>Low</td>
<td>−</td>
</tr>
<tr>
<td>1E11</td>
<td>IgM/κ</td>
<td>157.9</td>
<td>High</td>
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</tr>
<tr>
<td>10A2</td>
<td>IgM/κ</td>
<td>308.6</td>
<td>High</td>
<td>−</td>
</tr>
<tr>
<td>5G10</td>
<td>IgM/κ</td>
<td>354.4</td>
<td>Highest</td>
<td>+</td>
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</table>

<sup>a</sup>: normalized mean fluorescence intensity (MFI) of each hybridomas analyzed by flow cytometry; <sup>b</sup>: estimated from Fig.1; <sup>c</sup>: reactivity of purified Mabs with avian HEV viral ORF2 protein in IFA (Fig. 3).
Table 2. Inhibition of the binding of biotinylated Mabs (B-Mabs) to avian HEV ORF2 capsid protein

<table>
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<th>Biotin-labeled Mab</th>
<th>Inhibition by a non-labeled antibody (%)</th>
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<tr>
<td></td>
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<tr>
<td>7B2</td>
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<td>10A2</td>
<td>63.1</td>
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<td>5G10</td>
<td>8.3</td>
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Table 3. Detection of avian HEV RNA in sera and fecal swabs from chickens inoculated with mixtures of Mab and virus

<table>
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<tr>
<th>Groups</th>
<th>No. of positive sera (fecal swabs)/ total no. tested at indicated WPI</th>
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<tr>
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<tr>
<td>1E11</td>
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<td>0(0)/3</td>
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<tr>
<td>5G10</td>
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**Table 4.** Synthetic oligonucleotide primers used for avian HEV ORF2 fragments amplification

<table>
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<th>Primer</th>
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<th>Position (nt)</th>
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<td>GGGGGATCCAGTACATGTACGCCGGCGGCTG</td>
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<tr>
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<td>GGGGAATTCCAGTACATGTACGCCGGCGGCTG</td>
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<td>Avian HEVORF2-3L</td>
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<td>391-414</td>
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<td>Avian HEVORF2-4L</td>
<td>GGGGAATTCCAGTACATGTACGCCGGCGGCTG</td>
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</table>
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.

<table>
<thead>
<tr>
<th>Avian HEV ORF2</th>
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<tr>
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<td>Avian HEV ORF2-4</td>
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<td>110</td>
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</table>
Fig. 5.
Fig. 6.
Fig. 7.
CHAPTER 6. EGG WHITES FROM EGGS OF CHICKENS EXPERIMENTALLY INFECTED WITH AVIAN HEPATITIS E VIRUS CONTAIN INFECTIONOUS VIRUS BUT EVIDENCE OF COMPLETE VERTICAL TRANSMISSION IS LACKING

A paper submitted to Journal of General Virology

H. Guo¹, E. M. Zhou², Z. F. Sun³, X. J. Meng³

Abstract

Avian hepatitis E virus (avian HEV) is genetically and antigenically related to human HEV. Vertical transmission of HEV has been reported in humans, but not animals. In this study, we demonstrated that avian HEV RNA was detected by RT-PCR in egg white samples of eggs laid by chickens experimentally infected with avian HEV. To determine the infectivity of virus detected by RT-PCR in egg white, two groups of chickens were inoculated with either PCR-positive egg white sample or PCR-negative normal egg white sample. The results showed that the avian HEV in egg white remained infectious as evidenced by the appearance of viremia, fecal virus shedding and seroconversions in chickens inoculated with the PCR-positive egg white sample, but not in the chickens inoculated with the PCR-negative normal egg white sample. To assess the possibility of vertical transmission of avian HEV, two chickens were inoculated with an infectious avian HEV stock and housed with two

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roosters to produce embryonated eggs. Batches of embryonated eggs collected before and after virus inoculation were hatched and the hatched chicks were monitored for evidence of avian HEV infection. However, no virus was detected in samples collected from hatched chicks throughout this study, suggesting avian HEV could not complete the vertical transmission cycle. The finding of moderate amounts of infectious avian HEV in egg white might explain the significant level of IgG anti-HEV seroprevalence in healthy humans after repeated consumption of eggs containing avian HEV.

**Introduction**

Hepatitis E virus (HEV), which causes epidemic and sporadic self-liming acute hepatitis, is an important public health disease in many developing countries where sanitation condition is poor (Purcell & Emerson, 2001). Although, only sporadic cases were reported in the industrialized countries, anti-HEV antibodies are detected in 1–20% of the general population in these countries (Purcell & Emerson, 2005). Domestically acquired hepatitis E cases have recently been reported in industrialized countries including the United States, United Kingdom, French and Japan (Amon et al., 2006; Sadler et al., 2006; Mansuy et al., 2004; Mizuo et al., 2005). The mortality associated with HEV infection is generally as low as less than 1% in the general population, however, in pregnant women the mortality rate can reach up to 20% with unknown reason (Purcell & Emerson, 2001). HEV was also reportedly transmitted from infected mothers to their babies causing significant perinatal morbidity and mortality (Kumar et al., 2004; Singh et al., 2003; Kumar et al., 2001; Khuroo, et., 1995).

Hepatitis E is a zoonosis (Meng 2005; Tei et al., 2003; Tamada et al., 2004). Anti-HEV antibodies have been detected in a number of animal species including cattle, pigs,
chickens, sheep, goats, primates and rodents (Favorov et al., 1998; Tien et al., 1997; Arankalle et al., 1994; Kabrane-Lazizi et al., 1999), but thus far only two animal strains of HEV, swine HEV from pigs and avian HEV from chickens, have been definitively isolated and characterized (Meng et al., 1997; Haqshenas et al., 2001).

Cross-species infections have been demonstrated, as a US-2 strain of human HEV infected specific-pathogen-free (SPF) pigs and a swine HEV strain infected nonhuman primates (Halbur et al., 2001; Meng et al., 1998a). Swine have been considered as an important source of infection for swine veterinary workers and swine farmers (Zheng et al., 2006; Meng et al., 2002; Kasorndorkbua et al., 2005). However, the detection of a high level of anti-HEV prevalence in human populations who have no history of exposure to pigs suggests that multiple sources of exposure may exist (Meng et al., 2002).

Rodents could serve as a reservoir for HEV, since they are widely distributed in urban as well as rural environments (Emerson & Purcell, 2003), however, HEV has not yet been isolated from rodents even though wild-caught rodent populations are seropositive for HEV (Emerson & Purcell, 2003; Stoszek et al., 2006). HEV transmission via the consumption of undercooked or raw pig liver or deer meats has been reported, and could be a source for the observed high anti-HEV prevalence (Tei et al., 2003; Tamada et al., 2004).

Rhesus monkeys are frequently used as the animal model for HEV study, however infected pregnant monkeys failed to transmit the virus to offspring (Tsarev et al., 1995). An attempt to demonstrate vertical transmission of HEV from pregnant gilts to their pigs was unsuccessful (Kasorndorkbua et al., 2003). Avian HEV was first isolated from chickens with hepatitis-splenomegaly (HS) syndrome in the United States, and was shown to be genetically
and antigenically related to human HEV (Haqshenas et al., 2001, 2002; Huang et al., 2004). Avian HEV infection in chickens has been proven to be a good model system to study HEV replication and pathogenesis (Billam et al., 2005). The question of whether or not HEV can be vertically transmitted under controlled experimental conditions remains unknown.

In this study, we utilized the avian HEV and the chicken model system to investigate the feasibility of vertical HEV transmission. We demonstrated that avian HEV can be passed in egg whites in infected chickens and that the virus in the egg white is infectious. An experimental vertical transmission study was also attempted.

**Material and Methods**

**Avian HEV virus.** Since avian HEV can not be propagated *in vitro*, an infectious stock of avian HEV was produced by intravenously inoculating chickens with 200 μl of the avian HEV with a titer of $10^3$ 50% chicken infectious doses (CID$_{50}$) per ml (Sun et al., 2004). The avian HEV infectious stock containing $1 \times 10^4$ genome equivalent (GE)/ml in feces suspension was obtained and used for chicken inoculation in this study. An egg white sample containing avian HEV with a titer of $1 \times 10^4$ GE/ml were also used for infecting chickens.

**Detection and titration of avian HEV in egg white.** Intact eggs were collected from the chicken infection study aimed to generate avian HEV infectious stock. The detection of avian HEV virus in eggs was performed with a modified protocol that routinely used for avian influenza virus detection in the Veterinary Diagnostic Laboratory of Iowa State University. All eggs shells were washed with 70% ethanol before the separation of white and yolk. White samples were 1:3 diluted with 0.01M PBS buffer (pH 7.2) and centrifuged for 5 min at 5,000 g. 100 μl of the supernatants were used for RNA extraction with RNeasy Mini
Kit (Qiagen, CA). Total RNA was eluted with 20 µl DEPC-treated water. Reverse transcription (RT) with an avian HEV-specific primer was performed at 42 °C for 60 min in the presence of a master mixture consisting of 10 µl of total RNA, 0.25 µl of AMV reverse transcriptase (Promega), 1 µl of 10 µM antisense primers, 0.5 µl of RNase inhibitor, 4 µl of 5 × RT buffer, and 1 µl of 10 mM dNTP.

The resulting cDNA was amplified by a nested RT-PCR with Taq DNA polymerase (Promega) and two pairs of avian HEV specific primers targeting partial viral helicase gene as described previously (Sun et al., 2004) with the following modifications. The first round PCR parameters were 95 °C for 5 min, followed by 38 cycles of amplification at 94 °C for 1 min for denaturation, 52 °C for 1 min for annealing, and 72 °C for 1 min for extension, with a final incubation period at 72 °C for 7 min. For the second round PCR, 5 µl of the first round PCR products were used as the templates and the annealing temperature was increased to 53 °C and the amplification cycles were changed to 35. PCR was performed using the Mastercycler Personal (Eppendorf, NY). The final PCR products were examined on a 1.0% agarose gel containing 1.0 µg.ml⁻¹ ethidium bromide.

The genomic titer of avian HEV RNA in the positive egg white samples was determined similarly as the titration of swine HEV genomic RNA (Meng et al., 1998b). Briefly, positive egg white samples were 10-fold diluted with 0.01 M PBS buffer (pH 7.2) and each diluent was used for RNA extraction and nested RT-PCR as described above. One genome equivalent (GE) was defined as the number of viral genomes present in the highest 10-fold dilution that is positive by the nested RT-PCR (Meng et al., 1998b).
Specific-Pathogen-Free (SPF) chickens. Thirty-eight 22-week-old SPF hens and two 22-week-old SPF roosters (M&C Anderson Pullets, Sioux Rapids, IA), negative for antibodies to avian HEV, were used in this study. All chickens were tagged and divided into 3 groups. Group 1 and 2 each had 18 hens (ID#1–18 in Group 1, and ID#19 – #36 in Group 2) and chickens in each group were housed in 6 cages with 3 hens per cage. Group 3 contained 2 hens (ID#37, #38) and 2 roosters (ID#39, #40) and was housed together in a room.

Experimental inoculation and sample collection. All chickens in group 1 were each inoculated intravenously with 400 µl of the egg white sample #13 with a titer of $1 \times 10^4$ GE/ml. All chickens in group 2 were each similarly inoculated with 400 µl of a normal egg white sample (negative for avian HEV RNA by RT-PCR). The two hens in Group 3 were each inoculated intravenously with 400 µl an infectious stock of avian HEV with a genome titer of $1 \times 10^4$ GE/ml. Serum and fecal samples from each chicken were collected weekly for a total of 5 weeks and 100 µl of each sample were used for the detection of avian HEV viremia and fecal virus shedding by nested RT-PCR.

Egg hatching. Eggs were collected daily from Group 3 chickens starting from one week before virus inoculation to 5 weeks post inoculation (WPI). Eggs collected during the same week period were hatched as one batch in a RXI incubator with automatic egg turning (Lyon Electric Company, CA). Temperature and humidity were adjusted according to the manufacture’s instruction. A total of 6 batches of eggs were hatched. Each batch of chicks was housed separately and monitored daily for fecal virus shedding and viremia for one week before necropsy.
**Necropsy and pathology.** It has been shown that chicken liver is the major tissue that develops gross lesions after avian HEV infection (Billam et al., 2005). Necropsy was performed on 3 chickens from each of the Groups 1 and 2 at 1 to 4 WPI and on the remaining 6 chickens in each of the two groups at 5 WPI. Gross lesions in the livers were recorded by digital camera. Bile samples were collected from the necropsied chickens for the detection of viruses. The livers were also collected for virus detection from all chicks necropsied at seven days after hatch.

**Sequencing and sequence analysis.** Positive egg white samples were amplified by RT-PCR using the high-fidelity ProofStart DNA polymerase (Qiagen, CA). The final PCR products of 221bp were sequenced for both strands using ABI 3730 DNA Analyzer (Applied Biosystems, CA) at Iowa State University DNA Facility (Ames, Iowa) to confirm the identity of the viruses recovered from the inocula. Sequences were aligned and analyzed using the MegaAlign program (DNA STAR Inc., Madison, WI).

**ELISA assay.** Chicken serum samples from all the 14 chickens in Groups 1 to 3 that were continuously monitored for 5 weeks were tested for anti-avian HEV ORF2 antibody by an indirect ELISA as described (Guo et al., 2006). Briefly, ELISA plates were coated with avian HEV ORF2 antigens (100 ng/well) overnight at 4°C. After washing and blocking, 100 µl 1:100 diluted chicken sera were added to each well in duplicate and incubated for 1 hr. HRP-conjugated goat anti-chicken IgY-HRP conjugate (Jackson ImmunoResearch lab) was added as detecting antibody. O-phenylenediamine dihydrochloride (OPD) (Sigma) was used for colorimetric reaction.
Detection of serum lactate dehydrogenase (LDH). It has been demonstrated that avian HEV infection results in the elevation of serum LDH in infected chickens (Billam et al., 2005). Cytotoxicity LDH detection kit (Roche Applied Science, IN) was used to measure the LDH levels in sera from the chickens that were continuously monitored for 5 weeks after virus inoculation. Briefly, chicken sera were 1:10 diluted with freshly prepared 1% bovine serum albumin (BSA) in 100mM PBS (pH 7.5). Chicken LDH standard (Sigma) was also serially diluted and used to establish a standard curve. Freshly mixed catalyst and dye solution (100 μl) provided in the kit were added into each well of 96-well ELISA plate, followed by adding 100 μl of diluted serum samples and chicken LDH in duplicates. The 1% BSA diluent was added and used as a background control. After incubation at room temperature for 20 min, the reaction was stopped by adding 1N HCl (50 μl/well) and measured with ELISA reader at 490 nm. The absolute LDH activity in chicken sera was inferred from the LDH standard curve.

Statistical analysis: Statistical analysis was performed as described previously (Guo et al., 2006). P values of ≤0.05 were considered significant.

Results

Detection of avian HEV RNA in egg white

A total of 20 eggs (#1 to #20) were collected between 3 and 4 WPI from laying chickens during the course of an experiment aimed to generate an infectious stock of avian HEV. Egg whites were tested for avian HEV RNA by an avian HEV-specific nested RT-PCR assay. Five of the 20 eggs (#4, 13, 14, 16, 17) had detectable avian HEV RNA in egg white (Fig. 1A, B). Positive egg white samples were further titrated by a semi-quantitative
nested RT-PCR. Egg white sample #4 and #14 contained 100 GE/ml of avian HEV RNA (Fig. 1C, E), sample #13 had $1 \times 10^4$ GE/ml of avian HEV RNA (Fig. 1E), and sample #16 and #17 both had $1 \times 10^3$ GE/ml of avian HEV RNA (Fig. 1D). The PCR products amplified from the five egg white samples were sequenced, and sequence analyses confirmed that the viruses recovered from egg white were originated from the inocula. The avian HEV sequences from egg white sample #4, #13, #14, #16, and #17 were deposited in GenBank database with the accession numbers of DQ660974, DQ660975, DQ660976, DQ660977, DQ660978, respectively.

**Egg white contains infectious avian HEV**

Sera, feces and bile samples collected from the three groups of chickens were tested for the presence of avian HEV RNA by a nested RT-PCR. In group 1 chickens inoculated with PCR-positive egg white sample #13, viremia and fecal virus shedding were detected beginning from 3 WPI (Table 1). Virus was also detected variably in bile samples collected during necropsies in Group 1 chickens. However, viremia and virus shedding in feces or bile were not detected from Group 2 chickens, which were inoculated with normal egg white samples. Both hens in Group 3 that received an infectious stock of avian HEV had detectable viremia and fecal virus shedding starting at 1 WPI.

**Anti-avian HEV antibody response**

Prior to inoculation, all chickens in the 3 groups were seronegative for avian HEV. Anti-avian HEV IgG was detected at 2 WPI in both hens in Group 3 that were inoculated with an avian HEV infectious stock and remained seropositive throughout the study (Fig. 2). For the 6 chickens from Group 1 that were continuously monitored for 5 weeks, anti-avian
HEV IgG was detected at 4 and 5 WPI. As expected, anti-avian HEV antibody was not
detected from any of the chickens from Group 2, which were inoculated with normal egg
white samples.

**Serum LDH**

It has been shown that serum LDH enzyme was increased in chickens after
intravenous or oronasal inoculation with avian HEV (Billam *et al*., 2005). In this study, the
dynamics of serum LDH levels in the 6 continuously monitored chickens from Group 1
(inoculated with PCR-positive egg white sample #13) and in the 2 continuously monitored
hens from Group 3 (inoculated with avian HEV infectious stock as positive control) were
similar to that in the 6 continuously monitored chickens from Group 2 that were inoculated
with normal white samples, with the exception at 3 WPI, 1WPI, respectively (Fig. 3), in
which the LDH level in Group 1 and Group 3 was significantly higher than that in Group 2 (*P*
value of 0.015 and 0.017, respectively.).

**Attempt to detect avian HEV from hatched chicks**

As shown in table 2, 11 to 13 eggs were laid weekly by the 2 hens in Group 3. The
fertility of each batch of eggs reached from 91% to 100%. The hatchability of eggs collected
from 1 week before virus inoculation and 4 weeks after virus inoculation ranged from 82% to
85%. Eggs collected at 5 WPI had a relatively low hatchability (75%). Each batch of chicks
was daily monitored for viremia and fecal shedding by the nested RT-PCR. Bile and liver
samples collected from all chicks necropsied at 7 days of old were also subjected to the
nested RT-PCR. However, the hatched chicks were negative for avian HEV infection.

**Gross hepatic lesions**
Chickens necropsied weekly from Groups 1 and 2 had no visible gross subcapsular hemorrhage, which is the characteristic liver pathological change after experimental avian HEV infection (Billam et al., 2005). None of chicks necropsied at 7 days of old had observable liver abnormality.

**Discussion**

Avian HEV was isolated in the United States (Haqshenas, et al., 2001; Huang, et al., 2002) from chickens with HS syndrome, an emerging chicken disease in North America (Riddell, 1997; Ritchie & Riddell, 1991). This virus can be detected in liver tissues, bile, sera, feces from infected chickens (Haqshenas, et al., 2001; Huang, et al., 2002; Sun et al., 2004; Billam, et al., 2005). In this study, we found that avian HEV RNA is detected by RT-PCR in eggs from chickens experimentally infected with avian HEV (Fig. 1). Sequencing results confirmed that the virus detected in egg white is originated from the virus used for inoculation. Most importantly, we demonstrated that the virus in the egg white is still infectious as evidenced by the detection of viremia and fecal virus shedding in chickens inoculated with the PCR-positive egg white samples (Table 1). Seroconversion and slight elevation of serum level of LDH were also detected in the chickens inoculated with PCR-positive egg white sample, although they occurred later than those in chickens inoculated with an avian HEV infectious stock (Fig. 2, and Fig. 3).

Avian HEV is highly prevalent in the United States as anti-avian HEV antibodies have been found in approximately 71% of the chicken flocks in 5 states (Huang et al., 2002). Although we previously showed that avian HEV failed to infect 2 rhesus monkeys (6), avian
HEV does have the ability to cross species barriers and infect turkeys (Sun et al., 2004). Until now, there is no direct evidence as to whether avian HEV can infect human or not.

Chicken eggs are one of the most popular and highly nutritious foods, and eating raw eggs are common in many parts of the world (Doorduyn et al., 2006; Lievonen et al., 2004; Shiferaw et al., 2002; Sin et al., 2000). A multistate surveillance conducted in the United States between 1995 and 1996 had found that as high as 50% of the responders ate undercooked or raw eggs and more than 60% did not wash hands after cracking raw eggs and this habit has kept unchanged for years (Yang et al., 1998; Sara, et al., 2002). In addition, several foods contain raw eggs, such as meringue, hollandaise sauce, cookie dough, homemade mayonnaise, and Caesar salad dressing (Mazurek et al., 2005; Laura 2006). The finding of the infectious avian HEV in egg white and the significant percentages of individuals eating raw and undercooked eggs raised a question as to whether or not infected chicken eggs could be a potential source for the observed high seroprevalence of IgG anti-HEV in the general population in the industrialized countries, including the United States (Purcell & Emerson, 2005). Even if avian HEV does not infect humans, repeated exposures to the same foreign microorganism could cause the production of anti-HEV IgG antibody, especially since avian HEV could survive acidic stomach environment after its oral inoculation into chicken (Billam et al., 2005).

We have previously identified common antigenic epitopes in the ORF2 protein between avian HEV and human HEV (Guo et al., 2006). Western blot and ELISA results indicated that the avian HEV ORF2 capsid protein reacted with antisera against human HEV. Convalescent sera from SPF chickens experimentally infected with avian HEV also reacted
with the recombinant ORF2 capsid proteins of human HEV (Haqshenas et al., 2002). Therefore, it is possible that the IgG anti-HEV antibodies detected from healthy individuals in the United States and other industrialized countries could be the results of repeated exposures to avian HEV through the consumption of raw chicken eggs. Further studies are warranted to definitively determine if there is a correlation between IgG anti-HEV seroprevalence and egg-eating habits in humans.

Vertical transmission of human HEV was first reported in India, where six of eight infants from infected mothers had HEV infection (Khuroo et al., 1995). Approximately two-thirds of the infected pregnant women could have preterm deliveries (Kumar et al., 2004), and approximately 8% of HEV-RNA positive infants born to the infected mothers developed acute clinical disease that could cause early neonatal deaths (Kumar et al., 2001). The mortality rate among the HEV-infected pregnant women is up to 20% (Purcell & Emerson, 2001), however attempts to experimentally reproduce the severe hepatitis in pregnant rhesus monkeys and pregnant sows were unsuccessful and the infected pregnant animals failed to transmit virus to newborns (Tsarev et al., 1995; Kasorndorkbua et al., 2003).

Although avian HEV could be transmitted to egg white, it was not able to establish the vertical transmission, as no virus was detected in any samples collected from a total of 60 hatched chicks. Shivaprasad et al (1995) reported that the agent associated with the HS syndrome could not be isolated from chicken embryos with conventional routes of egg inoculation (Shivaprasad et al., 1995). In contrast, we previously showed that avian HEV could be successfully propagated in embryonated chicken eggs by intravenously inoculating 100 GE of avian HEV into 9-day-old embryonated chicken eggs (Meng et al.,
2006). Avian HEV was detected in bile and liver samples collected from hatched chicks necropsied at 2 to 3 days of age with very high GE titer (Meng et al., 2006). The virus had also been continuously detected in feces for at least 8 days after hatch.

In this study, instead of inoculating embryonated eggs, we directly inoculated each hen with 4000 GE of avian HEV, collected their eggs for hatching and then monitored the presence of virus in hatched chicks. In the five positive egg white samples from eggs laid by chickens inoculated with 1000 GE of avian HEV, two had an avian HEV genomic titer of 100 GE/ml and three had a titer of $\geq 1 \times 10^3$ GE/ml. A typical chicken egg contains more than 10 ml white, thus avian HEV virus can enter the egg white with a relatively high dose to infect chicken embryos and chicks. We proved that the virus in egg white remains infectious, but the stability of the infectivity during the hatching process is not known. Our result from this study that no virus could be detected in samples from the hatched chicks and our previous results (Meng et al., 2006) that avian HEV could be detected in samples from the chicks hatched from eggs that were intravenously inoculated with avian HEV at 9 days of embryonation (Meng et al., 2006) suggested that the infectivity of avian HEV in eggs before or at 9 days of embryonation has decreased to such an extent that the virus is unable to transmit further to chicks. The results from this study should help understand the transmission of HEV in humans.

Acknowledgements

This work is supported in part by a grant (to X.-J. M.) from the National Institutes of Health (AI 50611).
References


Table 1. Detection of avian HEV RNA in sera, fecal swabs and bile samples from inoculated chickens.

<table>
<thead>
<tr>
<th>Chicken groups</th>
<th>No. of positive sera (fecal swabs) / total no. tested at indicated WPI</th>
<th>No. of positive bile samples / total no. tested at indicated WPI</th>
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</thead>
<tbody>
<tr>
<td></td>
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<tr>
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<td></td>
<td>ND §</td>
<td>0/3</td>
</tr>
<tr>
<td>Group 3‡</td>
<td>0(0)/2</td>
<td>2(2)/2</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*, chickens were inoculated with an egg white sample positive for avian HEV RNA by RT-PCR; †, chickens were inoculated with normal egg white negative for avian HEV; ‡, only the two hens were inoculated with an avian HEV infectious stock; §, not done.
Table 2. Hatchability of eggs collected from hens experimentally infected with avian HEV

<table>
<thead>
<tr>
<th>Batch*</th>
<th>No. of eggs hatched</th>
<th>Hatchability</th>
<th>No. of eggs unhatched</th>
<th>Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dead embryos</td>
<td>No fertilization</td>
</tr>
<tr>
<td>-1</td>
<td>10</td>
<td>83%</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>85%</td>
<td>2</td>
<td>0</td>
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<tr>
<td>2</td>
<td>9</td>
<td>82%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>85%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>83%</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>75%</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

*: Eggs collected during the same week period were hatched in a batch before (-1 batch) and after (1 to 5 batch) hens were intravenously inoculated with an avian HEV infectious stock.
Figure 1: Detection & GE titration of avian HEV RNA in egg white. Panels A and B: 20 egg white samples were tested for the presence of avian HEV RNA by a nested RT-PCR assay that amplifies a 221 bp region in the avian HEV helicase gene. M: DNA marker; lane 1 to 20 are egg white samples #1 to #20, respectively. Panels C, D, and E: Genomic equivalent titer of avian HEV RNA determined by semi-quantitative RT-PCR. Total RNA was extracted from 100 µl of undiluted and 10 fold serially diluted (from left to right) egg white samples and amplified by the nested RT-PCR. Panel C: egg white sample #4; Panel D: egg white sample #16 (lane 1-5) and #17 (lane 6-10); Panel E: egg white sample #13 (lane 1-7) and #14 (lane 8-14).
Figure 2: Seroconversion to avian HEV IgG antibodies in chickens experimentally inoculated with PCR-positive egg white and with an avian HEV infectious stock. Sera from 14 chickens continuously monitored throughout the 5-week study were diluted at 1:100 and tested for the presence of anti-avian HEV ORF2 antibodies by ELISA. Recombinant avian HEV ORF2 antigen was coated at 100 ng/well on the ELISA plates. Each point represents the mean values (±S.E.M.) of the OD 490 nm obtained from duplicate ELISA results. Chickens #13 to #18 were inoculated with egg white sample #13 that is positive by PCR for avian HEV RNA. Chickens #31 to #36 were inoculated with a normal egg white sample that is negative for avian HEV RNA. Chickens #37 and #38 were inoculated with an avian HEV infectious stock as positive controls.
Figure 3: Serum level of LDH in infected and control chickens. The log mean LDH values at each WPI were generated from 6 continuously monitored chickens from Group 1 that were inoculated with an egg white sample #13 that was positive for avian HEV RNA, 6 continuously monitored chickens from Group 2 that were inoculated with a normal egg white sample that is negative for avian HEV RNA, and 2 continuously monitored hens from Group 3 that were inoculated with an avian HEV infectious stock. *, Statistical significance (P ≤0.05).
CHAPTER 7. GENERAL CONCLUSION

General discussion

Avian HEV infection is associated with the HS syndrome in chickens, an emerging disease in North American [1-4]. It has been demonstrated that avian HEV infection in chickens can serve as a valuable homologous small animal model for studying human HEV pathogenesis [5]. Avian HEV recombinant ORF2 protein was expressed in bacterial cells and used as target antigen for the detection of anti-avian HEV infection [2, 5-8]. But detailed analysis of this major viral antigen is lacking. In this thesis, the protective function of ORF2 protein of avian HEV and its epitope composition have been investigated. Since vertical transmission of HEV in both pregnant non-human primates and swine failed [9, 10], vertical transmission of avian HEV in chickens was also evaluated in hope of establishing a model to study some aspects of vertical transmission of human HEV.

Current ELISA methods using the recombinant ORF2 protein for HEV serological diagnosis can not differentiate avian HEV from swine HEV and possible human HEV infections, due to the cross-reactivity between the ORF2 proteins [6, 11]. In the first study, we found that at least one B-cell epitope in the antigenic domain I is shared among avian, human and swine HEV capsid proteins, and that at least one epitope in domain IV is shared between avian and human HEVs. However, epitopes in domain II are unique to avian HEV. Thus, peptide 8 from domain II could be used for the differential diagnosis of avian HEV infection by ELISA. Peptide 8 combing with peptide 1 and 4 can be used for differential diagnosis of human HEV infection. Peptide 1 combining with peptide 8, but not peptide 1 combining with peptide 4 can be used for differential diagnosis of swine HEV, as some field
swine HEV positive swine sera can react with peptide 4. It should be pointed out that a sufficient number of sera samples from different regions are needed for further evaluation of these peptide ELISAs before their actual applications.

In the second study, we demonstrated that chickens immunized with avian HEV ORF2 recombinant protein were able to produce high level of anti-ORF2 antibodies and were protected from avian HEV infection and/or the development of hepatitis. These findings showed that avian HEV ORF2 protein can be used as candidate vaccine for prevention of the emerging chicken HS disease. They also suggested avian HEV ORF2 protein is the major viral antigen that targeted by chickens, like its counterpart of human HEV [12-15]. Currently, virulent or very virulent strain of avian HEV has not been isolated or probably has not emerged yet [2], thus we couldn’t know whether the recombinant avian HEV ORF2 antigen used in this study can provide protection against virulent strains, although studies from human HEV had demonstrated that the immunization of ORF2 protein can provide protection against both homologous and heterogeneous challenges [16, 17] and convalescent sera induced by one genotype of human HEV can neutralize all four genotypes [14, 18, 19] and also one monoclonal antibody against human HEV ORF2 protein can neutralize at least two HEV genotypes [14].

Since neutralization epitope is the major component that determines the efficiency of human HEV ORF2 protein vaccine [12-14] and avian HEV is genetic and antigentic related to human HEV, avian HEV ORF2 protein probably also contains protective neutralization epitopes. Indeed, two independent neutralizing epitopes on avian HEV ORF2 capsid protein were identified in the third study by using neutralzing Mabs and recombinant proteins. This
finding provided evidence that protection against avian HEV infection by immunization with avian HEV ORF2 protein was probably due to the induced neutralizing antibodies. Although in the first study, we identified at least three epitopes on avian HEV ORF2 proteins, but our unpublished data showed neither of them is neutralizing epitope. Peptides containing these epitopes could not react with neutralizing Mabs, further supporting this conclusion.

In the fourth study, we are first to report the unexpected finding that avian HEV can be detected in eggs collected from chickens experimentally infected with avian HEV by nested RT-PCR. We further proved avian HEV in egg white is infectious. The finding of the infectious avian HEV in egg white and the fact that eating raw eggs is popular [20-24] and avian HEV is still of possible zoonotic concern prompted us to consider avian HEV infected eggs a new possible source of HEV seroprevalence in general population [25]. Although avian HEV could be transmitted to egg white, it failed to successfully establish the vertical transmission route, suggesting the infectivity of survived virus in egg white during hatch process is attenuated or completely lost.

**Recommendations for future research**

Although the combinations of peptides 1, 4 and 8 can be potentially used for differential diagnosis of HEV infection, the sensitivity of these peptide assays has not been evaluated. It also should be aware that synthetic peptides are such small molecules that may be not stable. The storage concentration, temperature and duration can significantly affect the peptide ELISA result. Conjugation of peptides with carrier molecules, like keyhole limpet hemocyanin (KLH) can be an approach to stabilize these peptides, but whether the
conjugation will affect the specificity and sensitivity of peptide ELISA assays needs to be tested.

Two different neutralization epitopes unique to avian HEV ORF2 protein were identified with four Mabs. However, it is likely that other neutralization epitopes not recognized by the four established Mabs may exist. These neutralization epitopes could be conserved among human and animal HEVs. Identification of these neutralization epitopes may thus provide a theoretical basis for applying avian HEV ORF2 protein as a candidate vaccine for prevention of human and animal HEV infections.

The fact that avian HEV in egg white is highly infectious and the possibility of avian HEV cross infection in human make it prudent to perform a large scale survey of the presence of infectious avian HEV in commercial eggs and investigate the egg eating habit in anti-HEV positive population. The obtained results may potentially provide further evidence for the possible food-borne zoonotic transmission of avian HEV.

To date, HEV has been found to be vertically transmitted only from pregnant mothers to fetuses causing death or disease in new-born infants [26-29]. Establishing an animal model for vertical transmission study would benefit the understanding of vertical pathogenesis. Thus, the continuing search of animal strains of HEV and investigating their vertical transmission route are warranted.

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