Genetic identification of avian hepatitis E virus (HEV) from healthy chicken flocks and characterization of the capsid gene of 14 avian HEV isolates from chickens with hepatitis–splenomegaly syndrome in different geographical regions of the United States

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Avian hepatitis E virus (HEV), a novel virus identified from chickens with hepatitis–splenomegaly (HS) syndrome, is genetically and antigenically related to human HEV. Recently, it was found that avian HEV antibody is also prevalent in healthy chickens. A prospective study was done on a known seropositive but healthy chicken farm to identify avian HEV isolates from healthy chickens. Fourteen chickens were randomly selected, tagged and monitored under natural conditions for 19 weeks. All 14 chickens had seroconverted to avian HEV antibody. None of the chickens had any signs of HS syndrome. Partial helicase gene and capsid gene sequences of avian HEV isolates recovered from a healthy chicken were determined and found to share 75–97 % nucleotide sequence identity with the corresponding regions of avian HEV isolates from chickens with HS syndrome. Thus far, only one strain of avian HEV from a chicken with HS syndrome has been genetically characterized for its capsid gene, therefore the capsid gene region of an additional 14 isolates from chickens with HS syndrome were also characterized. The capsid genes of avian HEV isolates from chickens with HS syndrome were found to be heterogeneous, sharing 76–100 % nucleotide sequence identity with each other. This study indicates that avian HEV is enzootic in chicken flocks and spreads subclinically among chickens in the United States and that the virus is heterogeneous.

INTRODUCTION

Hepatitis E virus (HEV), the causative agent of human hepatitis E, is an important human pathogen (Purcell, 1996; Reyes, 1997; Aggarwal & Krawczynski, 2000). The genome of HEV is about 7·2 kb in size and contains three open reading frames (ORFs) (Huang et al., 1992; Tsarev et al., 1992; Purcell, 1996; Reyes, 1997; Schlauder et al., 1998; Emerson et al., 2001). Although the mortality rate is generally low (less than 1%) in young adults, it can be up to 15–25% in infected pregnant women (Purcell, 1996; Hussaini et al., 1997; Reyes, 1997). Hepatitis E is an important public health concern in many developing countries (Huang et al., 1992; Arankalle et al., 1994; Purcell, 1996; Wang et al., 1999, 2000, 2002). Although only sporadic cases of acute hepatitis E have been reported in industrialized countries including the United States (Schlauder et al., 1998, 1999; Erker et al., 1999; Zanetti et al., 1999; Pina et al., 2000; McCrudden et al., 2000; Takahashi et al., 2001, 2002, 2003b; Clemente-Casares et al., 2003), a significant proportion of healthy individuals in industrialized countries are seropositive for HEV antibodies (Hsieh et al., 1999; Mast et al., 1997; Thomas et al., 1997; Meng et al., 1999, 2002; Meng, 2000a, b, 2003). Increasing evidence indicates that hepatitis E is a zoonosis (Meng et al., 1997, 1998, 1999, 2002; Kabrane-Lazizi et al., 1999; Favorov et al., 2000; Meng, 2000a, b, 2003; Nishizawa et al., 2003; Takahashi et al., 2003a, b; Tei et al., 2003).

Swine HEV, the first animal strain of HEV, was discovered and characterized from a pig in the United States in 1997 (Meng et al., 1997). Since then, many swine HEV isolates have been identified worldwide and shown to be genetically closely related to strains of human HEV (Chandler et al., 1999; Hsieh et al., 1999; Pina et al., 2000; Halbur et al., 2001; Haqshenas & Meng, 2001; Garkavenko et al., 2001;
Okamoto et al., 2001; van der Poel et al., 2001; Williams et al., 2001; Huang et al., 2002b; Kasorndorkbua et al., 2002; Wu et al., 2002; Takahashi et al., 2003a, b). Recently, another animal strain of HEV, avian HEV, was identified in the United States from chickens with hepatitis–spleenomegaly (HS) syndrome (Haqshenas et al., 2001) and shown to be antigenically and genetically related to human and swine HEVs (Haqshenas et al., 2001, 2002; Huang et al., 2002a). HS syndrome was first reported in 1991 in western Canada and has now been recognized in the United States (Ritchie & Riddell, 1991; Shivaprasad & Woolcock, 1995; Riddell, 1997). It has been shown that avian HEV shares approximately 50–60 % nucleotide sequence identity with the known human and swine HEVs and about 80 % sequence identity with the Australian chicken big liver and spleen disease virus (BLSV) (Payne et al., 1999; Haqshenas et al., 2001).

Our recent study showed that avian HEV antibody is highly prevalent in apparently healthy chicken flocks in the United States (Huang et al., 2002a). However, thus far avian HEV has only been genetically identified from chickens with HS syndrome (Haqshenas et al., 2001; Huang et al., 2002a; Sun et al., 2003). Therefore, it is important to genetically identify and characterize avian HEV from chickens without clinical disease. In addition, the extent of genetic variability of the ORF2 capsid gene of avian HEV isolates from chickens with HS syndrome is not known, as only one strain of avian HEV has been sequenced thus far for its ORF2 gene. Thus, it is also important to characterize the capsid genes of additional avian HEV isolates from chickens with HS syndrome in different geographical regions.

**METHODS**

**Clinical samples.** Bile samples used for the genetic characterization of the ORF2 capsid gene of avian HEV isolates were collected from 14 chickens with HS syndrome in California, Connecticut, New York and Wisconsin (Huang et al., 2002a). Clinical samples (serum and fecal materials) used for genetic identification and characterization of avian HEV isolates from healthy chickens were collected from a prospective study.

**Prospective study.** We have previously shown that avian HEV antibodies are highly prevalent not only in chicken flocks with HS syndrome but in healthy chicken flocks as well (Huang et al., 2002a). To identify genetically the virus responsible for the seropositivity in healthy chickens, we conducted a prospective study. Briefly, fourteen 12-week-old chickens were randomly selected from three healthy chicken flocks in a commercial farm in Virginia that had previously tested positive for avian HEV antibody (Huang et al., 2002a). Each of the 14 chickens was tagged and mixed with other chickens in the same flock. The 14 study chickens were housed and raised under the same natural conditions as the other chickens in the flocks. Weekly or biweekly fecal swabs and serum samples were collected from the 14 chickens for 19 weeks and the chickens were 30 weeks of age at the end of the prospective study. Both the serum and fecal samples were tested by RT-PCR for avian HEV RNA and the serum samples were also tested by an ELISA for avian HEV antibody.

**Primer design.** Primers used for genetic identification and characterization of avian HEV isolates were designed from the helicase gene region in ORF1 as well as from the ORF2 gene region based on multiple sequence alignments of the prototype avian HEV and other HEV isolates (Haqshenas et al., 2001; Huang et al., 2002a; Sun et al., 2003). For amplification of the helicase gene region, two nested sets of degenerate primers were used: external primer set AHEV F-1/SD, 5’-TGTATT(C)ACACCCCAACAG(A)ACGT(T)G-3’, and Helic R, 5’-CCTCA(G)TGCCAGCTA(T)ATGCACC-3’; and internal primer set AHEV F-2/SD, 5’-GCCACGGCTG(T)TTACACC(T)ACG(T)-GT-3’, and Helic R-2, 5’-GACCCA(G)GGA(G)TTGACTGCTT-3’. The sizes of expected PCR products for the first and second rounds were 452 bp and 386 bp, respectively.

**RT-PCR.** RNA was extracted with TriReagent (Molecular Research Center, Inc.) from 100 μl chicken fecal, bile or serum samples. Total RNA was resuspended in 12:25 μl DNase-free, RNase-free and protease-free water. Reverse transcription was performed at 42 °C for 60 min in the presence of a master mix consisting of 12:25 μl total RNA, 0:25 μl Superscript II reverse transcriptase (Invitrogen), 1 μl 10 μM antisense primer, 0.5 μl RNase inhibitor, 1 μl 0:1 M dithiothreitol, 4 μl 10 mM dNTPs. The resulting cDNA was amplified by PCR with appropriate primers and AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR reaction parameters consisted of an initial incubation at 95 °C for 9 min, followed by 39 cycles of 94 °C for 1 min, 42 °C for 1 min and 72 °C for 1:5 min, with a final incubation period at 72 °C for 7 min. The PCR products were examined on a 0.8 % agarose gel.

**ELISA to detect avian HEV antibody in chickens.** A truncated recombinant ORF2 capsid protein of avian HEV was expressed in Escherichia coli and purified by affinity chromatography using the BugBuster His-Bind Purification Kit (Novagen) (Haqshenas et al., 2002). The purified protein was used as an antigen to standardize an ELISA to detect avian HEV antibodies in chickens, as reported previously (Huang et al., 2002a). Sera from specific-pathogen-free (SPF) chickens were used as negative controls and convalescent sera from SPF chickens experimentally infected with avian HEV were included as positive controls. All sera were tested at least twice.

**Sequence and phylogenetic analyses.** The PCR products were excised from a 0.8 % agarose gel, purified using the GeneClean III kit (Qiogene, BIO 101 Systems) and directly sequenced at the Virginia Bioinformatics Institute Core Laboratory Facility with an automated DNA sequencer.

The PCR primer sequences were excised from the resulting sequences. Only 269 bp of the resulting 386 bp partial helicase gene and 172 bp of the resulting 242 bp partial ORF2 gene sequences of these avian HEV isolates were used for comparison with the available corresponding regions of BLSV and swine and human HEVs. The sequences of avian HEV isolates from a healthy chicken and 14 avian HEV isolates from chickens with HS syndrome were analysed and compared with the corresponding regions of the prototype avian HEV isolate, BLSV and selected strains of swine and human HEVs by the MacVector computer program (Oxford Molecular Inc.).

Phylogenetic analyses were conducted with the aid of the PAUP program (David L. Swofford, Smithsonian Institute, Washington, D.C.).
DC). The branch-and-bound search and mid-point rooting options with 1000 replicates were used to generate the phylogenetic trees. Phylogenetic analyses were performed on two different genomic regions: a 269 bp fragment of the ORF1 helicase gene, for which the sequence of BLSV is also known, and a 172 bp fragment of the ORF2 capsid gene.

RESULTS

Subclinical infection of chickens by avian HEV in a commercial chicken farm

Detection of avian HEV antibody in the majority of healthy chicken flocks in different geographical regions of the United States (Huang et al., 2002a) suggested that avian HEV infections are widespread and are not just limited to chickens with HS syndrome. To genetically identify avian HEV from healthy chickens, we performed a prospective study in a healthy commercial chicken farm. All 14 chickens from this healthy farm were seronegative at 12 weeks of age when the study began. The first chicken became seroconverted at the age of 13 weeks in building no. 1, followed within a few weeks by seroconversion of chickens in other cages housed in the same building (Table 1). Chickens in two other buildings began to seroconvert at about 14–16 weeks of age (Table 1). Most of the chickens in all three buildings had seroconverted by about 17–19 weeks of ages. By 21 weeks of age, all the 14 study chickens had seroconverted and remained seropositive at the end of the study (30 weeks of age). However, none of the 14 chickens had any clinical signs of disease consistent with HS syndrome. The course of antibody appearance in six representative chickens from this prospective study is presented in Fig. 1. The data from this prospective study indicate that avian HEV is enzootic in chicken flocks and spreads subclinically among chickens.

Genetic identification and characterization of avian HEV isolates from a healthy chicken flock

The weekly or biweekly faecal and serum samples were tested by RT-PCR for avian HEV RNA. Viraemia and faecal virus shedding were detected from the serum (1241-15S and 1241-16S) and faecal (1241-16F, 1229-16F and 1236-16F) samples of three healthy chickens (1241, 1229 and 1236) at 15 and 16 weeks of age, respectively, and from the serum and faecal samples of another healthy chicken (2553-26F, 2553-26S, 2553-27F and 2553-28F) for three consecutive weeks. The PCR products amplified from a representative healthy chicken (2553) were sequenced in two different regions: a 386 bp region in the helicase gene and a 242 bp region in the capsid gene. The sequences obtained were compared with those of BLSV and swine, human and avian HEV strains. Sequence analysis revealed that the sequences of the helicase gene region of the avian HEV isolates recovered from faecal and serum samples of the same healthy chicken (2553) in three different weeks were identical (data not shown). However, they shared 86% nucleotide sequence identity with the prototype avian HEV and 79–97% sequence identity with isolates recovered from chickens with HS syndrome. They also shared 75% identity with the Australian chicken BLSV and 51–54% sequence identity with selected known representative strains of swine and human HEVs (data not shown).

Sequence analysis based on the 172 bp fragment of the ORF2 gene revealed similar results. The ORF2 gene sequences of the isolates recovered from the faecal and serum

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<th>Bldg no.</th>
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Fig. 1. Seroconversion to IgG avian HEV antibody in six representative chickens from the prospective study. All chickens were seronegative at the beginning of the study, which was 12 weeks of age. All six representative chickens had seroconverted by 21 weeks of age and remained seropositive at the end of the study, which was 30 weeks of age. The ELISA cutoff value was 0·3 $A_{405}$. 

Table 1. Seroconversion to IgG avian HEV antibody in healthy chickens from a commercial chicken farm: a prospective study
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**Table 2.** Pairwise sequence comparison of the ORF2 capsid gene region of different avian HEV isolates and selected known representative strains of swine and human HEVs.

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samples of the same healthy chicken in three different weeks (2553-26F, 2553-26S, 2553-27F, 2553-27S, 2553-28F and 2553-28S) were 99–100% identical (Table 2). They shared 90% nucleotide sequence identity with the prototype avian HEV and 75–95% sequence identity with avian HEV isolates from chickens with HS syndrome. They also shared 47–51% sequence identity with selected known representative strains of swine and human HEVs (Table 2).

Phylogenetic analysis based on the partial ORF1 helicase gene and the partial ORF2 capsid gene indicated that the avian HEV isolates recovered from the faecal and serum samples of the same healthy chicken in three consecutive weeks clustered together (Fig. 2). The avian HEV isolates from a healthy chicken were genetically related to the prototype avian HEV and other avian HEV isolates associated with HS syndrome.

The capsid gene of avian HEV isolates recovered from chickens with HS syndrome in different geographic regions of the United States is heterogeneic

The extent of genetic variations of the important ORF2 capsid gene of avian HEV is not known since only one strain of avian HEV has thus far been characterized for its capsid gene (Haqshenas et al., 2001). By using RT-PCR, we successfully amplified the capsid gene region of 14 additional avian HEV isolates from the bile samples of chickens with HS syndrome in four different states. Sequence analysis revealed that these 14 additional avian HEV isolates from chickens with HS syndrome in different geographical regions shared 76–100% nucleotide sequence identity with each other and 48–54% sequence identity with known swine and human HEV strains (Table 2). The nucleotide sequence identities of the ORF2 gene region for the isolates recovered from chickens in the same state ranged from 80

**Fig. 2.** (A) Phylogenetic tree based on the nucleotide sequences of a 269 bp partial helicase gene region of avian HEV isolates and Australian chicken big liver and spleen disease virus (BLSV), as well as selected known representative strains of swine and human HEVs. (B) Phylogenetic tree based on the nucleotide sequences of a 172 bp partial ORF2 capsid gene region of avian HEV isolates and selected known representative strains of swine and human HEVs. The tree was constructed with the aid of the PAUP program. A branch-and-bound search with 1000 replicates and a mid-point rooting option was used to construct the trees. A scale bar representing the numbers of character state changes is proportional to the genetic distance. The avian HEV isolates recovered from a healthy chicken in the prospective study are shown in bold.
to 100% in California (CA077, CA242, CA518.3, CA518.5, CA697A, CA697B, CA697C, CA708A and CA889) and 96 to 98% in Wisconsin (W1318B, W1966B and W1966G). Even within the same farm, the partial ORF2 gene of different avian HEV isolates varied from 93 to 100% nucleotide sequence identity among the three isolates in a California farm (CA697A, CA697B and CA697C) and 99% identity between the two isolates in another California farm (CA518.3 and CA518.5) (Table 2).

Phylogenetic analysis of the ORF2 gene region revealed that avian HEV isolates from chickens with HS syndrome in different geographical regions are heterogeneous. Minor branches, indicating heterogeneity, exist among avian HEV isolates regardless of their geographical origins. Avian HEV isolates from both a healthy chicken and from chickens with HS syndrome are more distantly related to BLSV (Fig. 2).

**DISCUSSION**

Since avian HEV was only discovered about 2 years ago, little is known about its transmission and pathogenesis. Similar to swine and human HEVs, avian HEV is presumably transmitted through the faecal–oral route (Haqshenas et al., 2001, 2002; Huang et al., 2002a). Faeces from infected chickens appear to be the major source of virus. Chickens may get infected through direct contact with infected chickens or through faeces-contaminated feed or water. It is known that subclinical infection is the main outcome of swine HEV infection in pigs (Meng et al., 1997; Halbur et al., 2001; Meng, 2000a, b, 2003) and is also common for HEV infections in humans (Purcell, 1996; Mast et al., 1997; Thomas et al., 1997; Meng et al., 2002). We have previously shown that the majority of chicken flocks in the United States are seropositive for avian HEV antibodies (Huang et al., 2002a), suggesting that avian HEV infection is widespread among chickens. However, only sporadic cases of HS syndrome in chickens have been reported in the United States, and so far avian HEV isolates have only been genetically identified from chickens with HS syndrome. Here we report for the first time the genetic identification and characterization of avian HEV isolates from a clinically healthy chicken farm.

The data from the prospective study confirmed our previous seroepidemiological study showing that avian HEV is enzootic in chicken flocks (Huang et al., 2002a) and provided convincing evidence that subclinical avian HEV infection occurs in the majority of chickens in the United States. We demonstrated that, under natural conditions, chickens become infected at approximately 3–4 months of age. We speculate that the nature of subclinical infection in the majority of chickens is due to the relatively low doses of virus that can be transmitted among chickens via the faecal–oral route. It has been documented that human HEV infection in primates is dose-dependent: primates that received higher doses of human HEV developed biochemical and virological evidence of hepatitis, whereas primates that received lower doses had only subclinical infection, as evidenced by seroconversion to anti-HEV antibodies (Tsarev et al., 1994). This dose-dependent hypothesis may explain why there are only sporadic cases of HS syndrome in chickens, even though many chickens in the United States are infected with avian HEV (Huang et al., 2002a).

Sequence and phylogenetic analyses revealed that the capsid gene sequences of avian HEV isolates from chickens with HS syndrome in four different states were heterogeneous, regardless of their geographical origins. This observation is consistent with reports on swine and human HEVs, which are also heterogeneous. Sequence analyses based on the ORF1 helicase gene and ORF2 capsid gene regions showed that the sequences of the avian HEV isolates recovered from a healthy chicken in three different weeks were nearly identical. Phylogenetic analysis also revealed that the avian HEV isolates recovered from a healthy chicken clustered together and were genetically related to, but different from, the prototype avian HEV and avian HEV isolates recovered from chickens with HS syndrome.

In summary, the genetic identification of avian HEV isolates from a healthy chicken farm and the demonstration of subclinical avian HEV infection in chicken flocks further complicates the causal relationship between avian HEV infection and HS syndrome in chickens. Although we believe that avian HEV infection is dose-dependent and that only chickens infected with higher doses of the virus develop HS syndrome, we cannot rule out the possibility that the avian HEV isolate identified from healthy chickens may represent an avirulent strain and that the subclinical infections may be caused by an avirulent strain of avian HEV. Further studies are warranted to fully characterize this avian HEV isolate recovered from a healthy chicken, both experimentally and genetically.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


