The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and chicken big liver and spleen disease virus


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We recently identified a novel virus, designated avian hepatitis E virus (avian HEV), from chickens with hepatitis–splenomegaly (HS) syndrome in the USA. We showed that avian HEV is genetically related to swine and human HEVs. Here we report the antigenic cross-reactivity of the putative open reading frame 2 (ORF2) capsid protein of avian HEV with those of swine and human HEVs and the Australian chicken big liver and spleen disease virus (BLSV). The region encoding the C-terminal 268 amino acid residues of avian HEV ORF2 was cloned into expression vector pRSET-C. The truncated ORF2 protein was expressed in E. coli as a fusion protein and purified by affinity chromatography. Western blot analysis revealed that the avian HEV ORF2 protein reacted with antisera against the Sar-55 strain of human HEV and with convalescent antiserum against swine HEV and the US2 strain of human HEV, as well as with antiserum against BLSV. Convalescent sera from specific-pathogen-free chickens experimentally infected with avian HEV also reacted with the recombinant capsid proteins of swine HEV and Sar-55 human HEV. Antiserum against the US2 human HEV also reacted with recombinant ORF2 proteins of both swine HEV and Sar-55 human HEV. The antigenic cross-reactivity of the avian HEV putative capsid protein with those of swine and human HEVs was further confirmed, for the most part, by ELISA assays. The data indicate that avian HEV shares certain antigenic epitopes in its putative capsid protein with swine and human HEVs, as well as with BLSV. The results have implications for HEV diagnosis and taxonomy.

Introduction

Human hepatitis E, caused by the hepatitis E virus (HEV), is an important public health disease in many developing countries (Arankalle et al., 1994, 1995, 1999; Purcell, 1996; Reyes, 1997; van Cuyck-Gandre et al., 1997; Wang et al., 1999, 2000). The disease is also endemic in some industrialized countries, including the USA (Schlauder et al., 1998, 1999; Erker et al., 1999; Zanetti et al., 1999; Pina et al., 2000; McCrudden et al., 2000; Meng, 2000a, b; Schlauder & Mushahwar, 2001; Takahashi et al., 2001). The genome of HEV is a single-stranded positive-sense RNA molecule of about 7.5 kb and contains three open reading frames (ORFs) and two short non-coding regions at its 5’ and 3’ ends (Purcell, 1996; Reyes, 1997; Emerson et al., 2001; Haqshenas & Meng, 2001). The primary route of HEV transmission is faecal–oral and the disease has a relatively high mortality rate, up to 20%, in infected pregnant women (Purcell, 1996; Hussaini et al., 1997; Reyes, 1997). The first animal strain of HEV, swine HEV, was identified and characterized from a pig in the USA (Meng et al., 1997). Swine HEV has now been identified from pigs in many other countries and shown to be genetically closely related to strains of human HEV, especially to those strains from the same geographic regions as swine HEV (Chandler et al., 1999; Hsieh et al., 1999; Pina et al., 2000; Garkavenko et al.,...
Interspecies transmission of HEV has been documented: swine HEV infects non-human primates and a US strain of human HEV infects pigs (Meng et al., 1998b; Halbur et al., 2001). We recently showed that pig handlers are at increased risk of zoonotic HEV infection (Meng et al., 1999, 2002). Taken together, these data indicate that swine are animal reservoirs for HEV. However, a relatively high level of anti-HEV antibody prevalence has also been reported in normal US blood donors who have no history of contacting pigs (Mast et al., 1997; Thomas et al., 1997; Meng et al., 2002), suggesting that other sources of exposure also exist.

Recently, we discovered yet another animal strain of HEV, avian HEV, from chickens with hepatitis–spleenomegaly (HS) syndrome in the USA, and showed that avian HEV is also genetically related to human and swine HEVs (Haqshenas et al., 2001). HS syndrome has been reported in chickens from Canada and the USA and is characterized by increased mortality in broiler breeder hens and laying hens of 30–72 weeks of age (Ritchie & Riddell, 1991; Tablante & Vaillancourt, 1994; Tablante et al., 1994; Julian, 1995). The infected chickens usually show regressive ovaries, red fluid in the abdomen, enlarged liver and spleen and up to 20% drop in egg production (Ritchie & Riddell, 1991; Shivaprakash & Woolcock, 1995; Riddell, 1997). We have genetically characterized avian HEV and shown that it shares approximately 50–60% nucleotide sequence identity with human and swine HEVs and approximately 80% sequence identity with the Australian chicken big liver and spleen disease virus (BLSV) (Payne et al., 1999; Haqshenas et al., 2001). The putative ORF2 capsid protein of avian HEV consists of 606 amino acid residues, which is about 60 amino acids shorter than that of other HEV strains. Based on sequence analysis, conserved regions of amino acid sequences among the ORF2 capsid proteins of avian, swine and human HEVs have been identified (Haqshenas et al., 2001). The objective of this study was to determine whether common antigenic epitopes exist among avian, swine and human HEVs, as well as the Australian chicken BLSV.

**Methods**

- **Avian HEV.** The source of avian HEV used in this study was a positive bile sample from a chicken with HS syndrome, which contained about $10^7$ genomic equivalents (GE) of avian HEV per ml of bile (Haqshenas et al., 2001).

- **Antiserum against avian, swine and human HEVs and BLSV.** To generate avian HEV antiserum, specific-pathogen-free (SPF) chickens, 1 day old (Charles River Laboratories), were inoculated intravenously with a diluted bile sample containing approximately $10^6$ GE of avian HEV. The inoculated SPF chickens excreted avian HEV in the faeces and developed viraemia ( Fang and others, unpublished data). The convalescent serum collected from SPF chickens experimentally infected with avian HEV at 30 days post-inoculation was used as the avian HEV antiserum in this study. The antiserum against the Sar-55 strain of human HEV had been generated previously by immunizing two SPF pigs with the baculovirus-expressed capsid protein of Sar-55 human HEV (Meng et al., 1997). The antiserum against swine HEV and the US2 strain of human HEV were convalescent sera from SPF pigs experimentally infected with the respective virus strains (Halbur et al., 2001; Williams et al., 2001). The chicken polyclonal antiserum against Australian BLSV was a gift from Christine Payne (Murdoch University, Australia) (Payne et al., 1999).

- **Recombinant ORF2 capsid proteins of swine and human HEVs.** The putative capsid proteins of Sar-55 human HEV and swine HEV were expressed in a baculovirus (Tsarev et al., 1993; Robinson et al., 1998; Meng et al., 2002). The recombinant ORF2 capsid proteins of Sar-55 human HEV and swine HEV used in this study were kindly provided by Drs Suzanne Emerson and Robert Purcell (National Institutes of Health, Bethesda, Maryland, USA).

- **Cloning of the truncated ORF2 gene of avian HEV.** The C-terminal 804 bp sequence of avian HEV ORF2 was amplified by RT–PCR with a sense primer (5′ GGGGGATCCAGTACATGTCGCCCCTGCTG 3′) and an antisense primer (5′ GGGGATCCCTTGGGTGGTAGGGAATG 3′). To facilitate subsequent cloning steps, a BamHI site and an EcoRI site (underlined) were introduced at the 5′ ends of the sense and antisense primers, respectively. Proofreading Pfu DNA polymerase (Stratagene) was used for the PCR amplification. The amplified fragment was purified and digested with BamHI and EcoRI restriction enzymes and cloned into the pRSET-C expression vector (Invitrogen). The truncated ORF2 gene was cloned in-frame with the sequence encoding the Xpress epitope (24 nucleotides in size) (Invitrogen) located upstream of the multiple cloning site of the expression vector. The Xpress epitope was expressed as a fusion protein with the truncated avian HEV ORF2 protein and was detected by an anti-Xpress antibody (Invitrogen). E. coli DH5α cells were transformed with the recombinant plasmids. The recombinant expression vector was isolated with a Qiagen Plasmid Mini Kit (Qiagen) and confirmed by restriction enzyme digestions and DNA sequencing.

- **Expression and purification of the truncated ORF2 capsid protein of avian HEV.** The recombinant plasmids were transformed into E. coli strain BL21(DE3)pLysS, which had been engineered to produce T7 RNA polymerase. Expression of the fusion protein was driven by a T7 promoter upstream of the Xpress epitope sequence. By using the pRSET-C vector, the recombinant fusion protein was tagged with six tandem histidine residues at the N-terminus, which have a high affinity for ProBond resin (Invitrogen). The transformed cells were grown in SOB broth containing 50 µg/ml ampicillin and 25 µg/ml chloramphenicol. Expression of the fusion protein was induced by the addition of 1 mM IPTG for 4–6 h at 37°C. The fusion protein was expressed in E. coli strain BL21(DE3)pLysS as inclusion bodies. To confirm that the expressed recombinant fusion protein contained the Xpress epitope, the crude bacterial lysates were separated on a 12% polyacrylamide gel containing 0·1% SDS and transferred on to a nitrocellulose membrane (Osmonics). The immobilized protein on the membrane was incubated with a 1:5000-diluted horseradish peroxidase (HRP)-conjugated monoclonal antibody (Invitrogen) against the Xpress epitope. The immunocomplexes were detected using 4-chloro-l-naphthol (Sigma).

The fusion protein was purified from 50 ml of bacterial cultures using the ProBond Purification System (Invitrogen) based on the affinity of ProBond resin for the His-tagged recombinant fusion protein. Bacterial cells were lysed with guanidinium lysis buffer (6 M guanidinium hydrochloride, 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8) and clarified by centrifugation at 30000 g for 10 min. The supernatant was added to the resin pre-equilibrated with the binding buffer and gently agitated for 10 min at room temperature to allow the
fused protein to bind to the resin. The protein-bound resin was serially washed six times with denaturing binding buffer (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride) twice each at pH 7.8, 6.0 and 5.3, respectively. The protein was eluted in elution buffer (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 4.0) and the fractions containing the highest concentration of protein were determined using the Bio-Rad protein assay reagent kit (Bio-Rad). Five µg of the purified protein was analysed by SDS–PAGE. The presence of the purified fusion protein was confirmed using the monoclonal antibody against the Xpress epitope, as described above.

**Western blot analysis.** Western blot analysis was used to determine whether the truncated ORF2 capsid protein of avian HEV shares antigenic epitopes with those of human and swine HEVs and BLSV. The purified truncated ORF2 protein of avian HEV was separated by SDS–PAGE (250 ng/lane) and transferred on to a nitrocellulose membrane. The blots were cut into separate strips and then incubated for 1 h in blocking solution (20 mM Tris–HCl, pH 7.5, 500 mM NaCl) containing 2% BSA. The strips were then incubated overnight at room temperature with a 1:100 dilution of antiserum against either avian, swine or human HEV in Tris-buffered saline (20 mM Tris–HCl, pH 7.5, 500 mM NaCl) containing 0.05% Tween 20 (TBST) and 2% BSA, or with a 1:1000 dilution of antiserum against BLSV diluted in TBST. Pre-inoculation or pre-immunization swine and chicken sera were diluted 1:100 and used as negative controls. The strips were washed twice with TBST and once with TBS. After a 3 h incubation with HRP-conjugated goat anti-swine IgG (1:2000; KPL) or HRP-conjugated rabbit anti-chicken IgY (1:2000; Sigma), the strips were washed again as described above and the immunocomplexes were detected using 4-chloro-1-naphthol.

To confirm further the antigenic cross-reactivity of the various capsid proteins, approximately 250 ng of recombinant capsid protein of swine HEV and Sar-55 human HEV were separated by SDS–PAGE and blotted on to a nitrocellulose membrane. The membrane strips were incubated with antisera against either avian, swine or human HEV. Serum dilution, incubation and washing steps were carried out as described above.

**ELISA assays.** To assess further the antigenic cross-reactivity of avian, swine and human HEVs, we performed ELISA assays with different antigens and antisera. The 96-well ELISA plates (Dynex Technologies) were coated for 2 h at 37 °C with purified recombinant ORF2 antigens of avian, swine and human HEVs. Each antigen was adjusted to a concentration of 2 µg/ml in sodium carbonate buffer (pH 9.6). The plates were incubated with blocking solution (10% fetal bovine serum and 0.5% gelatin in washing buffer) to reduce non-specific binding. The antisera against each virus was diluted 1:200 in blocking solution. Pre-incubation sera from SPF pigs and chickens were used as negative controls. The plates were incubated with diluted antisera for 30 min at 37 °C and then washed four times with washing solution (PBS, pH 7.4, containing 0.05% Tween 20). The HRP-conjugated secondary antibodies were added and incubated at 37 °C for 30 min. The plates were again washed four times with washing solution and the immunocomplexes were detected using 2,2′-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid. The optical density (OD) values were measured at 405 nm.

**Sequence analysis and computer prediction.** The predicted amino acid sequence of the truncated ORF2 protein of avian HEV was compared with the corresponding regions of swine and human HEVs using the MacVector program (Oxford Molecular). Hydrophathy and antigenicity plots of the amino acid sequences were determined according to the Kyte–Doolittle (Kyte & Doolittle, 1982) and Welling (Welling et al., 1985) methods using the MacVector computer program.

### Results

#### Expression of the truncated ORF2 capsid protein of avian HEV

The recombinant plasmid containing the truncated ORF2 gene of avian HEV was transformed into *E. coli* strain BL21(DE3)pLysS. On induction with IPTG, the truncated ORF2 protein of avian HEV was expressed in bacterial cells with a very high yield. The expressed protein was observed on the SDS–PAGE gel at the expected size of approximately 32 kDa (Fig. 1A). Samples taken at different time points revealed that the maximum expression occurred approximately 4–6 h after induction with IPTG (Fig. 1A). Western blot analysis using a monoclonal antibody against the Xpress epitope of the 32 kDa fusion protein confirmed the expression of the recombinant fusion ORF2 protein of avian HEV (Fig. 1B). Although the bacterial cells used in this study contain the pLysS plasmid to minimize the background expression, background protein expressions were still observed (Fig. 1A). The recombinant fusion protein was present as inclusion bodies in the bacterial cells and was shown to be insoluble (Fig. 1A). The protein purification method was efficient as about 6 mg of purified protein were obtained from approximately 50 ml of bacterial cultures.

### Antigenic cross-reactivity among avian, swine and human HEVs and BLSV demonstrated by Western blot analysis

Western blot analysis demonstrated that the purified truncated ORF2 protein of avian HEV reacted strongly with the convalescent sera from SPF chickens experimentally infected with avian HEV. The purified truncated avian HEV ORF2 protein did not react with pre-inoculation control SPF chicken sera (Fig. 2). The avian HEV ORF2 protein also reacted strongly with antisera against the Australian chicken BLSV. The truncated ORF2 protein of avian HEV also reacted with antisera against the capsid protein of Sar-55 human HEV and with convalescent swine sera against the US2 human HEV and swine HEV, although the reaction signals were relatively weak compared with the homologous avian HEV antisera (Fig. 2). Swine HEV antisera reacted strongly with the recombinant swine HEV ORF2 antigen. Antisera against the US2 and Sar-55 strains of human HEV also reacted with the recombinant swine HEV capsid protein. The recombinant Sar-55 antigen reacted strongly with the antisera to Sar-55 human HEV, and to a lesser extent, with heterologous antisera against swine, avian and the US2 human HEVs. Antisera against the avian HEV also reacted with swine HEV capsid protein.

### Antigenic cross-reactivity among avian, swine and human HEVs demonstrated by ELISA

The antigenic cross-reactivity among avian, swine and human HEVs was further confirmed, for the most part, by...
Fig. 1. (A) Expression of the truncated avian HEV ORF2 protein containing the C-terminal 268 amino acid residues. Lanes 1–6, SDS–PAGE analysis of bacterial cell lysates at 0, 1, 2, 3, 4 and 6 h after IPTG induction, respectively; lane 7, soluble proteins in the supernatant of cell lysates; lane 8, insoluble proteins after solubilization in SDS; lane 9, SDS–PAGE analysis of 5 µg of the purified recombinant fusion protein. (B) Western blot analysis of bacterial cell lysates at 0 (lane 1) and 3 h (lane 2) after IPTG induction and of the purified protein (lane 3), using monoclonal antibody against the Xpress epitope located at the N terminus of the fusion protein. The expected product of approximately 32 kDa is indicated by arrows.

Fig. 2. Western blot analysis of antigenic cross-reactivity among avian, swine and human HEVs and BLSV. About 250 ng per lane of the indicated recombinant ORF2 proteins were separated by SDS–PAGE and transferred to a membrane. The sera were diluted 1:100 in blocking solution before being added to the membranes. The experiments were repeated twice, with similar results. Arrowheads show the expected size of the avian HEV truncated ORF2 protein; arrows show the swine and human HEV recombinant ORF2 proteins. αHEV, avian HEV; sHEV, swine HEV; Sar-55, Sar-55 strain of human HEV; US2, US2 strain of human HEV.

The OD value obtained from cross-reacting avian HEV ORF2 antigen with Sar-55 human HEV antiserum was approximately twice that recorded when the negative control serum was used. However, the OD value obtained from reactions between avian HEV antigen and antiserum to the US2 human HEV was similar to that obtained with the negative control sera, in contrast to the positive cross-reaction demonstrated by Western blotting (Fig. 2).

Sequence analyses and computer predictions

The ORF2 gene of avian HEV shares approximately 48–49% amino acid sequence identity with the corresponding genes of swine HEV and the US2 and Sar-55 strains of human HEV (Haqshenas et al., 2001). The ORF2 gene of swine HEV displays approximately 98% and 91% amino acid sequence identity with those of the US2 and Sar-55 strains of human HEV, respectively (Meng et al., 1997, 1998b). The ORF2 gene of Sar-55 human HEV shares approximately 91% amino acid sequence identity with that of the US2 human HEV (Meng et al., 1997; Schlauder et al., 1998). Amino acid sequence alignment of the truncated avian HEV ORF2 protein with the corresponding regions of swine HEV and the Sar-55 and US2 strains of human HEV revealed that the most conserved region of the truncated ORF2 protein is located at its N terminus (Fig. 4), which is rich in hydrophilic amino acids (Fig. 5). By using the Welling method to predict antigenic domains of the protein (Welling et al., 1985), four hydrophilic and antigenic regions were identified at amino acid positions 50–72, 121–135, 217–228 and 252–262 of the truncated avian HEV ORF2 protein (Figs 4 and 5). The relative positions of these putative antigenic domains (I, II, III and IV) were similar for all HEV strains. The most conserved domain appeared to be domain I (Fig. 4). Domain IV appears to be less antigenic in swine and human HEVs than in avian HEV (Fig. 5).
Antigenic cross-reactivity of HEV strains

Discussion

The genomic organization of avian HEV is similar to that of human and swine HEVs, and avian HEV is also morphologically similar to human HEV (Haqshenas et al., 2001). The complete ORF2 and ORF3 genes and partial ORF1 gene consisting of a partial helicase gene and the complete RdRp gene of avian HEV have been cloned and sequenced. Sequence analysis has revealed that avian HEV shares approximately 48–60% nucleotide sequence identity with the corresponding regions of human and swine HEVs and about 80% nucleotide sequence identity with BLSV (Haqshenas et al., 2001). The ORF2 capsid protein of human HEV is very immunogenic and induces neutralizing antibodies against HEV (Purcell, 1996; Riddell et al., 2000; Schofield et al., 2000). Riddell et al. (2000) expressed the C-terminal 267 amino acid residues of a truncated ORF2 protein of a human HEV in a bacterial expression system and showed that the region spanning amino acids 394–457 of the ORF2 protein participated in the formation of immunodominant epitopes on the surface of HEV particles. This truncated ORF2 protein has been successfully used in an ELISA assay to detect HEV infection in humans (Anderson et al., 1999). It has been shown that the C terminus of the ORF2 protein is masked when expression of the complete ORF2 protein is carried out in a bacterial expression system (F. Li et al., 1997). Im et al. (2001) showed that a homodimer of a truncated ORF2 protein of a human HEV lacking the C-terminal 57 amino acid residues was able to induce protective immunity against HEV infection in primates. It has also been shown that the N-terminal 112 amino acids and the C-terminal 50 amino acids of ORF2 are not involved in the formation of virus-like particles (T. Li et al., 1997). Therefore, we expressed and characterized the C-terminal 268 amino acid residues of the avian HEV ORF2 protein, which correspond to

Fig. 3. Antigenic cross-reactivity among avian, swine and human HEVs and BLSV demonstrated by ELISA. Each antiserum was tested in duplicate against each antigen.

Fig. 4. Amino acid sequence alignment of the C-terminal 268 residues of avian HEV ORF2 with the corresponding regions of swine HEV and the US2 and Sar-55 strains of human HEV. Predicted putative antigenic domains, corresponding to the domains in Fig. 5, are indicated in boxes. The deletions are indicated by minus (–) signs.
the C-terminal 267 amino acid residues of the human HEV ORF2 protein expressed by Riddell et al. (2000).

Western blot analysis revealed that, as expected, antiserum to each virus reacted strongly with its homologous viral antigen. The antigenic cross-reactivity of antisera with heterologous antigens was relatively weak compared with that of homologous antigens. For the most part, the antigenic cross-reactivity among avian, swine and human HEVs and BLSV demonstrated by Western blot analysis was further confirmed by ELISA (Fig. 3). In the ELISA, avian HEV antigen reacted with antisera to avian HEV, BLSV, swine HEV and Sar-55 human HEV but not with antiserum to US2 human HEV or negative control sera. In addition, the OD values from reactions of avian HEV antigen with antisera to Sar-55 human HEV and swine HEV were lower than those obtained from the reciprocal reactions between avian HEV antiserum and the Sar-55 human HEV and swine HEV antigens (Fig. 3). The differences in OD values in reciprocal ELISA reactions are probably due to the variation in different antiserum titres. In addition, the Sar-55 human HEV and swine HEV recombinant antigens are both truncated 55 kDa ORF2 proteins, whereas the avian HEV ORF2 protein is a truncated 32 kDa fusion protein, and this may also contribute to the difference in OD values. It has been reported that swine HEV infection can be detected by ELISA using Sar-55 human HEV antigen (Meng et al., 1998a, 1999, 2002), indicating that some common antigenic epitopes exist between swine HEV and human HEV. Similarly, detection of anti-HEV antibodies has also been reported in chickens using human HEV antigen (Tien et al., 1997). These serological results indicate that these animal strains of HEV are anti-
Antigenic cross-reactivity of HEV strains


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References


BLSV has been shown to cause a big liver and spleen disease in Australian chickens with similar pathological lesions to those seen in chickens with HS syndrome in the USA (Payne et al., 1999; Haqshenas et al., 2001). Based on a short available sequence in the helicase gene, BLSV was also found to be genetically related to human HEV (Payne et al., 1999). Here we have demonstrated that chicken polyclonal antisera against BLSV reacts strongly with the recombinant ORF2 protein of avian HEV in both Western blot and ELISA, indicating that BLSV is also antigenically related to avian HEV. Additional sequence information from BLSV is needed to characterize the taxonomic relationship among BLSV, avian HEV and other HEV strains.

Based on the computer prediction and comparison of the antigenicity and hydrophilicity plots, it appears that the N-terminal region of the truncated ORF2 protein contains conserved antigenic epitopes among different HEV strains. Schofield et al. (2000) showed that neutralizing monoclonal antibodies recognized linear epitope(s) located between amino acids 578 and 607 of the ORF2 protein of a human HEV. The genomic region in avian HEV corresponding to this defined neutralizing epitope is located within the truncated ORF2 protein of avian HEV, which reacted with antisera to human and swine HEVs. It will now be important to determine whether antisera against avian HEV can neutralize swine and human HEVs and vice versa. The observed antigenic cross-reactivity among HEV strains will make it difficult to interpret HEV seroepidemiological data in humans, especially since swine HEV (and perhaps avian HEV) is zoonotic.


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