Identification of an antigenic domain in the N-terminal region of avian hepatitis E virus (HEV) capsid protein that is not common to swine and human HEVs

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The antigenic domains located in the C-terminal 268 amino acid residues of avian hepatitis E virus (HEV) capsid protein have been characterized. This region shares common epitopes with swine and human HEVs. However, epitopes in the N-terminal 338 amino acid residues have never been reported. In this study, an antigenic domain located between amino acids 23 and 85 was identified by indirect ELISA using the truncated recombinant capsid proteins as coating antigens and anti-avian HEV chicken sera as primary antibodies. In addition, this domain did not react with anti-swine and human HEV sera. These results indicated that the N-terminal 338 amino acid residues of avian HEV capsid protein do not share common epitopes with swine and human HEVs. This finding is important for our understanding of the antigenicity of the avian HEV capsid protein. Furthermore, it has important implications in the selection of viral antigens for serological diagnosis.

Avian hepatitis E virus (HEV) is the principal causal agent of hepatitis–splenomegaly syndrome or big liver and spleen disease in chickens. It leads to an increase in mortality (1–4 %), a decrease in egg production (20–40 %) and an enlarged liver and spleen in both broiler breeder and laying hens from 30 to 72 weeks of age (Meng & Shivaprasad, 2013; Ritchie & Riddell, 1991). In addition, serological and molecular investigations have shown that subclinical infection with avian HEV is common (Peralta et al., 2009; Huang et al., 2002; Sun et al., 2004). Avian HEV belongs to the genus Hepeviridae within the new family Hepeviridae. It is a non-enveloped, single-stranded positive-sense RNA virus related genetically and antigenically to human and swine HEVs (Meng, 2010). To date, HEV in mammals has been classified into four major genotypes with only one serotype (Meng, 2010). Genotypes 1 and 2 have only been detected in humans, whereas genotypes 3 and 4 HEV are found in swine and humans (Cooper et al., 2005; Lu et al., 2006). Avian HEV has three major genotypes and also has only one serotype (Bilic et al., 2009).

The full-length avian HEV genome is approximately 6.6 kb, consisting of two non-coding regions and three partially overlapping ORFs. Avian HEV capsid protein is encoded by ORF2, which consists of 606 aa residues and shares 48–49 % aa identity with swine and human HEVs (Bányai et al., 2012; Haqshenas et al., 2001; Hsu & Tsai, 2014; Marek et al., 2010; Zhao et al., 2010). The HEV capsid protein contains immunodominant epitopes and is responsible for the induction of the protective humoral immune response (Huang et al., 2002; Li et al., 2004; Purdy et al., 1993; Riddell et al., 2000; Zhang et al., 2001; Zhao et al., 2013). Antigenic domains of avian, swine and human HEV capsid proteins have been...
determined in order to facilitate vaccine design and sero-epidemiological investigations (Guo et al., 2006, 2008; Khudyakov et al., 1993, 1994a, b; Li et al., 1994, 1997). For example, four antigenic domains (I–IV) and two neutralizing epitopes have been identified in the C-terminal 268 aa residues (aa 338–606) of avian HEV capsid protein, of which domain I (aa 389–410) is common to avian, human and swine HEVs and domain II (aa 477–492) is unique to avian HEV (Guo et al., 2006; Haqshenas et al., 2002; Zhou et al., 2008). Therefore, using the C-terminal 268 aa residues of avian HEV capsid protein as an antigen for serological diagnosis cannot differentiate avian HEV infection from human and swine HEV infections (Haqshenas et al., 2002). However, when only domain II (aa 477–492) is used as a coating antigen, the ELISA can differentiate these infections (Guo et al., 2006). In addition, three antigenic domains (aa 12–92, aa 143–222 and aa 221–373) in the N-terminal 392 aa residues (aa 1–392) of human HEV capsid protein were identified by indirect ELISA using synthetic overlapping peptides as coating antigens and anti-HEV human sera as the primary antibodies (Khudyakov et al., 1999). However, little is known about epitopes in the N-terminal 338 aa residues (aa 1–338) of avian HEV capsid protein. An important issue is whether the N-terminal 338 aa residues of avian HEV capsid protein contain antigenic domains that are common to human, swine and avian HEVs, or alternatively unique to avian HEV.

To identify antigenic domains located in the N-terminal 338 aa residues of avian HEV capsid protein, five truncated overlapping fragments were designed based on the hydrophilicity, antigenicity and surface probability plots of the N-terminal 358 aa residues using the Kyte–Doolittle and Jameson-Wolf methods with the Protean program of the Lasergene software (DNASTAR) (Fig. 1a). The five fragments were named aORF2-F (aa 23–358), aORF2-F-1 (aa 23–183), aORF2-F-2 (aa 85–243), aORF2-F-3 (aa 110–287) and aORF2-F-4 (aa 159–358), respectively (Fig. 1a). The genes of the five truncated fragments were amplified by reverse transcriptase PCR (RT-PCR), with five primer pairs (Table S1, available in the online Supplementary Material), using avian HEV stock virus isolated from China (CaHEV, GenBank accession no. GU954430) as a template.

To obtain corresponding regions of swine HEV, the amino acid sequences of avian HEV capsid proteins were compared with different genotypes of HEV using the CLUSTAL W method of the MEGALIGN program of the Lasergene software (DNASTAR). Based on the alignment (Fig. S1), the truncated protein containing the N-terminal 391 aa residues (aa 23–413) of swine HEV (genotype 4) capsid protein was designed (named sORF2-F). The gene of sORF2-F was amplified by RT-PCR using a swine HEV stock from the Shandong province of China (CHN-SD-sHEV genotype 4, GenBank accession no. KF176351) as a template with sORF2-FF and sORF2-FR primers (Table S1).

To facilitate subsequent cloning steps, a BamHI site and a XhoI site were introduced at the 5' end of the forward and reverse primers, respectively (Table S1). The six genes were amplified, digested with BamHI and XhoI restriction enzymes and cloned into a bacterial expression vector. The resulting recombinant proteins were used as coating antigens in the ELISA to determine the specificity and sensitivity of the assay.

![Fig. 1](http://vir.sgmjournals.org)  
**Fig. 1.** Hydrophilicity plot, antigenic index and surface probability were predicted by DNASTAR software using Kyte–Doolittle and Jameson-Wolf methods. (a) Schematic diagram of the N-terminal 358 aa residues of CaHEV capsid protein and four truncated fragments. (b) The corresponding region of CHN-SD-sHEV.
enzymes and subcloned into a pET-28a (+) vector containing an amino-terminal His-Tag (Novagen). After confirmations by double enzyme digestion and sequencing (data not shown), the six positive recombinant plasmids were transformed into *Escherichia coli* strain Rosetta BL21(DE3) plysS for expression. The blank pET-28a (+) vector was used as a control. All six recombinant proteins were expressed in Luria–Bertani (LB) medium containing 100 μg kanamycin ml⁻¹ and 0.1 mM IPTG and were purified using a Ni-NTA resin column (JinSiTe) according to the manufacturer’s instructions. After expression and purification, SDS-PAGE analysis showed that these six proteins (aORF2-F, aORF2-F-1, -F-2, -F-3, -F-4 and sORF2-F) were expressed as insoluble forms and purified with the predicted sizes of 37 kDa, 22 kDa, 22 kDa, 24 kDa, 26 kDa and 40 kDa, respectively (Fig. 2a). Western blotting showed that these six proteins reacted specifically with the anti-His monoclonal antibody (JinSiTe) (Fig. 2b). The six purified recombinant proteins were renatured by dialysis in renaturation buffer (1 mM EDTA, pH 7.2 PBS, urea at a gradient of 6 mM, 4 mM and 2 mM). These renatured six proteins were used as antigens to react with anti-avian, swine and human HEV antibodies using indirect ELISA and Western blotting.

Ten specific-pathogen-free (SPF) chickens were inoculated intravenously (i.v.) with 200 μl of CaHEV infectious virus stock containing 10⁴ genomic equivalents (GE) ml⁻¹. From these chickens, anti-avian HEV chicken sera was collected at 28 or 35 days post-inoculation. To produce swine HEV antisera, five pigs (7 weeks old), all serologically negative by ELISA of sera (Wantai Biological Pharmacy) and negative for RNA by RT-PCR of faeces (Wang et al., 2014) were inoculated i.v. with 10⁴ GE ml⁻¹ of CHN-SD-sHEV stock. The inoculated pigs excreted swine HEV in the faeces and developed viraemia (unpublished data). Anti-swine HEV pig sera were collected at 21 days post-inoculation. Animal experiments were conducted under the guidelines of the Institutional Animal Care and Use Committee of Northwest A&F University. In addition, 150 clinical swine HEV antisera which were confirmed in a previous study (Wang et al., 2014) were collected from herds in Shandong province, China. For human HEV antisera, five sera from human patients with acute-phase hepatitis E and five sera from healthy blood donors were obtained from Shandong Agriculture University Hospital (Zhang et al., 2009). The ten human sera were tested for anti-HEV antibodies with a commercial ELISA kit (Wantai Biological Pharmacy). The results showed that the OD₄₅₀ values of the five human sera with hepatitis E ranged from 1.14 to 1.85. The values of the five healthy donor sera were from 0.089 to 0.187.

The chicken, swine and human sera described above were tested using the developed indirect ELISAs using the C-terminal 268 aa residues of avian and swine HEV capsid proteins (aORF2-C and sORF2-C) (Wang et al., 2014; Zhao et al., 2013) or peptide 1 mocking domain I as coating antigens (Guo et al., 2006). The results showed that the sera from post-inoculated chickens, swines, clinically positive swines and humans cross-reacted with aORF2-C, sORF2-C and peptide 1 (Table S2). This further confirmed that the C-terminal 268 aa residues of avian and swine HEV capsid proteins and domain I contain common epitopes to avian, swine and human HEVs.

Subsequently, the above sera were used to identify antigenic domains in the N-terminal 358 aa residues of avian HEV capsid protein and search for common epitopes to avian, swine and human HEVs. The sera were used to detect aORF2-F, aORF2-F-1, -F-2, -F-3, -F-4 and sORF2-F by indirect ELISA. The sera from pre-challenge SPF chickens, swines and five clinically negative humans were used as negative controls. The indirect ELISA was performed as described previously (Zhao et al., 2013) with the following modifications. Briefly, ELISA plates (Nunc-Immunoplate) were coated separately with aORF2-F,
Table 1. Antigenic cross-reactivity of antisera from chickens, pigs and humans with five overlapping truncated capsid proteins of avian HEV and one truncated capsid protein of swine HEV

Five fragments from avian HEV were designed based on the antigenic analysis of the N-terminal 358 amino acid residues of CaHEV capsid protein (Fig. 1a). The fragment from swine HEV contains the N-terminal 413 amino acid residues of CHN-SD-sHEV capsid protein (Fig. 1b).

<table>
<thead>
<tr>
<th>Number of samples</th>
<th>Source</th>
<th>OD_{450} values of indirect ELISA against different antigens*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>aORF2-F</td>
</tr>
<tr>
<td>Antisera from SPF chickens experimentally infected with CaHEV</td>
<td>10 Pre-sera</td>
<td>0.156 ± 0.043</td>
</tr>
<tr>
<td></td>
<td>10 Positive</td>
<td>1.012 ± 0.137</td>
</tr>
<tr>
<td>Antisera from pigs experimentally infected with CH-SD-sHEV</td>
<td>5 Pre-sera</td>
<td>0.183 ± 0.024</td>
</tr>
<tr>
<td></td>
<td>5 Positive</td>
<td>0.167 ± 0.043</td>
</tr>
<tr>
<td>Antisera from pigs and humans naturally infected with HEV</td>
<td>150 Pig positive</td>
<td>0.202 ± 0.095</td>
</tr>
<tr>
<td></td>
<td>5 Human negative</td>
<td>0.136 ± 0.049</td>
</tr>
<tr>
<td></td>
<td>5 Human positive</td>
<td>0.119 ± 0.023</td>
</tr>
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</table>

*Numbers are the mean ELISA OD_{450} values ± SD.
†Values in bold type are considered positives.

aORF2-F-1, -F-2, -F-3, -F-4 and sORF2-F proteins. The plates were then washed with PBST [0.01 M PBS, pH 7.2, containing 0.5% Tween 20 (v/v)] and blocked with blocking buffer [2.5 % dry milk (w/v) in PBST]. The chicken, swine and human sera were diluted in blocking buffer containing 10 % E. coli Rosetta BL21(DE3) pLysS lysate and were added the wells in duplicate. After washing again, goat anti-chicken, anti-pig and anti-human immunoglobulins G (IgG)-HRP (BoaoSeng Company; 1:5000 dilution in 0.01 M PBS, pH 7.2) were added to respective wells. After a final washing step, tetramethylbenzidine (TMB) [made up of solution A: 205 mM potassium citrate (pH 4.0); and solution B: 41 mM tetramethylbenzidine; A:B (v/v) = 39:1] was added to each well and incubated for 15 min at room temperature. The colour reaction was stopped by adding 3 M H_{2}SO_{4} and OD_{450} values were read using an automatic ELISA plate reader (Universal 192 Micro plate Reader, EL8000; Bio-Rad).

The ten sera from chickens infected experimentally with CaHEV reacted with aORF2-F and aORF2-F-1, but not with aORF2-F-2, -F-3, -F-4 or sORF2-F (Table 1). In addition, the sera from pigs infected experimentally with CHN-SD-sHEV and clinically positive swine and human sera reacted with sORF2-F, but not with aORF2-F-1, -F-2, -F-3 or -F-4 (Table 1). These results suggested that: (1) there is only one antigenic domain (aa 23–85) in the N-terminal 358 aa residues of avian HEV capsid protein (Fig. 1); (2) the antigenic domain of aa 23–85 is unique to avian HEV; and (3) the N-terminal 391 aa residues of swine HEV capsid protein can be used as an antigen to detect anti-human HEV antibodies. None of the pre-challenge sera from chickens, swines or clinically negative human sera reacted with any of the six proteins (Table 1). Western blotting was used to confirm the reaction between the six proteins and these sera and the results were in agreement with those of indirect ELISA (Fig. S2). Amino acid sequence alignment of the N-terminal 358 aa residues of avian HEV capsid protein with the corresponding regions of swine and human HEVs revealed that the most deleted region is located at the N terminus of avian HEV capsid protein (Fig. S1). In addition, by using the Kyte-Doolittle and Jameson-Wolf methods to analyse the hydrophilicity, antigenicity and surface probability plots of the regions of CaHEV and CHN-SD-sHEV capsid proteins, more antigenic domains were predicted in regions of CHN-SD-sHEV capsid protein than in CaHEV (Fig. 1b). Based on the alignment and antigenic prediction of the N-terminal 358 aa residues in the present study and the C-terminal 268 aa residues of HEV capsid proteins in a previous study (Haqshenas et al., 2002), it is indicated that the antigenicity of the N-terminal region of capsid protein is more variable than the C-terminal region between avian and human or swine HEVs.

The antigenic properties of the human HEV capsid protein have been studied using overlapping synthetic peptides and expression of truncated proteins (Khudyakov et al., 1993, 1994a, b, 1999; Li et al., 1994, 1997). To date, six antigenic domains (I–VI) have been identified in the human HEV capsid protein, and most antigenic epitopes have been identified in domains I and VI located in the N- and C-terminal region, respectively (Khudyakov et al., 1999). For the avian HEV capsid protein, four antigenic domains (I–IV) have been predicted in the C-terminal 268 aa residues. Subsequently, four epitopes have been identified in the aa
339–389 region, which are different from the previously identified antigenic domains I–IV (Dong et al., 2011; Guo et al., 2006; Haqshenas et al., 2002). We named the aa 339–389 region as domain V. In the present study, another antigenic domain located in the N-terminal aa 23–85 region was identified and named domain VI. Thus, through the analysis of antigenic domains of avian HEV capsid protein, six antigenic domains have been identified to date.

In a previous study, it was determined that the C-terminal 268 aa residues of avian HEV capsid protein contained epitopes common to avian, human and swine HEVs (Guo et al., 2006). In addition, the locations of antigenic domains and epitopes in the region of avian and human HEV capsid proteins were similar. These findings suggested that the antigenic structure of the C-terminal 268 aa residues in avian HEV capsid protein is similar to human HEV (Dong et al., 2011). In contrast to the C-terminal region, the distributions of antigenic domains in the N-terminal 358 aa residues of avian capsid proteins are different from the corresponding region of human HEV. This is based on the identification of one unique antigenic domain in the N-terminus of avian HEV capsid protein and three antigenic domains in the corresponding region of human HEV capsid protein (Khudyakov et al., 1999).

In conclusion, an antigenic domain between aa 23 and 85 located in the N-terminus of avian HEV capsid protein was identified in the present study. This domain was shown to be unique to avian HEV. This information helps us understand the antigenicity of the avian HEV capsid protein and has important implications in the selection of viral antigens for serological diagnosis.

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