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

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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 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.
proteins were used in the ELISA. The purity of eluted proteins was confirmed by SDS-PAGE. The concentration was determined using a Protein assay kit (Bio-Rad). Eluted proteins were collected and purified by electroelution. Briefly, ORF2 inclusion body protein purified by electroelution. Briefly, ORF2 inclusion body protein was excised and the copper stain was removed from each peptide-immunized group were tested immediately after the booster injection with peptide-KLH in Freund’s incomplete adjuvant was given 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

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

**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 electrophoresis. Briefly, ORF2 inclusion body protein efficient 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 electrophoresis 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.
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 A490 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 antichicken IgY-HRP 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).

![Fig. 2. Expression and purification of avian HEV ORF2 protein. (a) Lanes 1–2, SDS-PAGE analysis of two bacterial cell clone lysates at 4 h 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 electroelution purification. The expected size of the truncated avian HEV ORF2 protein is 32 kDa.](http://vir.sgmjournals.org)

Titrations of rabbit antisera

Titrations of rabbit anti-peptide antisera (RS1–RS9) was performed by ELISA. Serum titres were defined as the highest serum dilution that gave an A490 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 (A490 1.280 and 1.111, respectively) and RS5 reacted with peptides 5 and 1 (A490 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 (A490 1.136), 8 (A490 1.026) and 9 (A490 1.239) but not with peptide 7 (A490 0.062). RS8 and RS9 cross-reacted with peptide 2 (A490 0.631 and 0.617, respectively) and to each other (A490 1.020 and 1.185, respectively). In contrast, RS7 only reacted with peptide 7 (A490 0.964) and did not react with peptide 2 (A490 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 0.02, 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.

**Cross-reactivity of anti-swine and anti-human HEV antisera with avian HEV ORF2 antigen and peptides**

Convalescent sera from pigs experimentally infected with swine HEV or human HEV and sera from field pigs naturally infected with swine HEV were used to assess the reactivity with avian HEV ORF2 antigen and peptides 1–4 and 8. As shown in Table 1, sera from pigs experimentally infected with swine HEV cross-reacted with avian HEV ORF2 and peptide 1, but not with peptides 2–4 and 8. In contrast, sera from pigs experimentally infected with human HEV (US-2 strain) cross-reacted with avian HEV ORF2 antigen and peptides 1 and 4. These convalescent swine serum samples are known to react with human HEV ORF2 antigen (Halbur et al., 2001). Sera from two negative control pigs did not react with these 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).

**Fig. 4.** Cross-reaction of rabbit anti-sera (RS1–9) with nine peptides. Rabbit sera were diluted at 1/1000 and used in 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.

**Fig. 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<0.05).
**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>
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<tr>
<td></td>
<td>ORF2</td>
<td>Peptide 1</td>
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<td>Convalescent swine antisera from experimentally infected pigs</td>
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<td>2</td>
<td>Negative sera 0·132 ± 0·004 0·083 ± 0·001 0·803 ± 0·001 0·078 ± 0·004 0·072 ± 0·006 0·047 ± 0·008</td>
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<td>6</td>
<td>Swine HEV 1·186 ± 0·104† 0·564 ± 0·054 0·134 ± 0·016 0·110 ± 0·032 0·149 ± 0·024 0·078 ± 0·015</td>
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<td>6</td>
<td>Human HEV 1·747 ± 0·208 0·762 ± 0·042 0·156 ± 0·028 0·112 ± 0·022 0·429 ± 0·049 0·060 ± 0·014</td>
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<tr>
<td>Antisera from naturally infected pigs</td>
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<td>3</td>
<td>Canada, negative 0·104 ± 0·003 0·090 ± 0·023 0·082 ± 0·019 0·083 ± 0·017 0·079 ± 0·019 0·041 ± 0·005</td>
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<tr>
<td>1</td>
<td>Canada, positive 1·145 0·574 0·106 0·103 0·141 0·050</td>
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<td>2</td>
<td>Canada, positive 1·222 ± 0·563 0·168 ± 0·014 0·130 ± 0·021 0·110 ± 0·013 0·131 ± 0·009 0·045 ± 0·004</td>
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<td>3</td>
<td>China, negative 0·132 ± 0·011 0·154 ± 0·029 0·134 ± 0·017 0·135 ± 0·034 0·149 ± 0·054 0·056 ± 0·002</td>
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<td>China, positive 2·814 ± 0·112 0·641 ± 0·036 0·457 ± 0·003 0·419 ± 0·012 0·605 ± 0·071 0·043 ± 0·001</td>
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<td>Korea, positive 0·448 0·120 0·100 0·081 0·097 0·051</td>
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<tr>
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<td>Korea, positive 0·973 0·458 0·111 0·128 0·398 0·053</td>
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<td>1</td>
<td>Thailand, negative 0·145 ± 0·031 0·141 ± 0·040 0·149 ± 0·013 0·136 ± 0·019 0·123 ± 0·027 0·046 ± 0·011</td>
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<tr>
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<td>Thailand, positive 1·224 0·180 0·203 0·177 0·173 0·040</td>
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<td>1</td>
<td>Thailand, positive 1·538 0·534 0·182 0·129 0·112 0·043</td>
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<td>1</td>
<td>Thailand, positive 1·786 0·523 0·128 0·147 0·370 0·050</td>
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<td>USA, negative 0·178 ± 0·010 0·102 ± 0·019 0·121 ± 0·046 0·119 ± 0·065 0·116 ± 0·052 0·041 ± 0·005</td>
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<td>USA, positive 0·635 ± 0·137 0·140 ± 0·017 0·132 ± 0·042 0·130 ± 0·052 0·133 ± 0·009 0·076 ± 0·003</td>
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<tr>
<td>Convalescent antisera from chickens experimentally infected with avian HEV</td>
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<tr>
<td>4</td>
<td>Pre-sera 0·180 ± 0·012 0·054 ± 0·009 0·053 ± 0·007 0·048 ± 0·007 0·053 ± 0·008 0·060 ± 0·007</td>
<td></td>
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<tr>
<td>6</td>
<td>Positive 3·000 ± 0·000 2·328 ± 0·434 0·069 ± 0·013 0·064 ± 0·001 2·322 ± 0·248 1·289 ± 0·218</td>
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*The numbers are the mean ELISA absorbance values ± standard deviation.
†The values in bold are considered positives.

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 equally efficient for detecting 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).

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