Identification of two neutralization epitopes on the capsid protein of avian hepatitis E virus

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Avian hepatitis E virus (avian HEV) is genetically and antigenically related to human HEV, the causative agent of hepatitis E. To identify the neutralizing epitopes on the capsid (ORF2) protein of avian HEV, four mAbs (7B2, 1E11, 10A2 and 5G10) against recombinant avian HEV ORF2 protein were generated. mAbs 7B2, 1E11 and 10A2 blocked each other for binding to avian HEV ORF2 protein in a competitive ELISA, whereas 5G10 did not block the other mAbs, suggesting that 7B2, 1E11 and 10A2 recognize the same or overlapping epitopes and 5G10 recognizes a different one. The epitopes recognized by 7B2, 1E11 and 10A2, and by 5G10 were mapped by Western blotting between aa 513 and 570, and between aa 476 and 513, respectively. mAbs 1E11, 10A2 and 5G10 were shown to bind to avian HEV particles in vitro, although only 5G10 reacted to viral antigens in transfected LMH cells. To assess the neutralizing activities of the mAbs, avian HEV was incubated in vitro with each mAb before inoculation into specific-pathogen-free chickens. Both viraemia and faecal virus shedding were delayed in chickens inoculated with the mixtures of avian HEV and 1E11, 10A2 or 5G10, suggesting that these three mAbs partially neutralize avian HEV.

INTRODUCTION

Hepatitis E virus (HEV) is a single-stranded, positive-sense RNA virus. The viral genome contains three open reading frames (ORF1, -2 and -3), which encode, respectively, non-structural viral proteins, the capsid protein and a small protein that may be involved in modulating cell signalling (Emerson & Purcell, 2003; Tam et al., 1991). HEV infection causes frequent endemic and rare epidemic outbreaks of acute hepatitis E in many developing countries. 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 USA (Amon et al., 2006; Mansuy et al., 2004; Mizuo et al., 2005; Sadler et al., 2006).

After the isolation of the first animal strain of HEV, swine HEV, isolated from pigs in the USA in 1997 (Meng et al., 1997), another animal strain of HEV, avian HEV, was recently identified in the USA from chickens with hepatitis–splenomegaly (HS) syndrome (Haqshenas et al., 2001). Pathological lesions characteristic of HS syndrome have been reproduced in specific-pathogen-free chickens experimentally infected with avian HEV (Billam et al., 2005). Avian HEV can be transmitted into chicken eggs and remain infective, although direct evidence of its infection in humans is lacking (Guo et al., 2007a). Avian HEV has been classified as a putative fifth genotype within the genus Hepevirus (Emerson et al., 2004), 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 (Huang et al., 2004), it shares 50–60 % nucleotide sequence identity, common antigenic epitopes and other significant functional features with these mammalian HEVs (Guo et al., 2006; Haqshenas et al., 2002; Huang et al., 2004).

An anti-HEV humoral immune response is sufficient to confer protective immunity (Bryan et al., 1994; Tsarev et al., 1994), and the ORF2 capsid protein is the most immunogenic and is responsible for the induction of a protective humoral immune response (Li et al., 2004; Purdy et al., 1993; Riddell et al., 2000). As a result, the ORF2 capsid protein is the target of vaccine designs, and recombinant proteins expressed in either Escherichia coli or insect cells have been used for evaluation of vaccine efficacy (Emerson & Purcell, 2001; Im et al., 2001; Li et al., 2005; Purcell et al., 2003; Zhang et al., 2001). A major
neutralizing epitope has been identified on the strain Sar-55 HEV ORF2 capsid protein (Schofield et al., 2000, 2003), and monoclonal antibodies (mAbs) raised against this epitope were able to neutralize two mammalian HEV genotypes (Emerson et al., 2006).

Four major antigenic domains have been predicted in the C-terminal 268 aa of the avian HEV capsid protein (Haqshenas et al., 2002). By using nine synthetic peptides derived from these four antigenic domains, we previously demonstrated that B-cell epitopes in antigenic domain I are shared among avian, human and swine HEV capsid proteins, epitopes in domain IV are shared between avian and human HEVs, and one epitope in domain III is unique to avian HEV (Guo et al., 2006). 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 were either located in another region(s) or were non-linear. To identify the potential neutralizing epitopes, we generated mAbs against avian HEV ORF2 protein and then determined their ability to neutralize avian HEV. In addition, a series of truncated avian ORF2 recombinant proteins was expressed to map the epitopes recognized by these mAbs.

METHODS

Antigen preparation. The recombinant pRESET-C plasmid containing the truncated ORF2 capsid gene of avian HEV was produced as described previously (Haqshenas et al., 2002). Recombinant avian HEV ORF2 protein was expressed as an inclusion body with an estimated molecular mass of 32 kDa and purified according to methods described elsewhere (Guo et al., 2006). Purified protein (10 mg) was precipitated with aluminum sulfate, as described previously (Schick et al., 1987).

Hybridoma production. Four BALB/c mice were injected intraperitoneally with 100 µg prepared avian HEV ORF2 capsid protein followed by two booster doses at 2 week intervals. Mice were bled before each injection and 2 weeks after the second and third injections. Two weeks after the third immunization, mice with the highest antibody titres were selected as the spleen cell donors. Four days prior to the fusion, the donor mice were given a final booster injection of 100 µg antigen. Hybridomas were produced by the fusion of spleen cells with SP2/0 murine myeloma cells with standard polyethylene glycol-mediated fusion methods.

Indirect ELISA. Hybridomas were screened for secreting mAbs using an indirect ELISA, essentially as described previously (Guo et al., 2006). Supernatants from hybridoma cells with an absorbance value three 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 their stability and specificity. A total of four mAbs was obtained with IgM isotypes with a κ-light chain, as determined using a mouse mAb isotyping kit (Bio-Rad).

To determine the specificity of the mAbs, four other recombinant proteins – human Sar-55 HEV ORF2 (Robinson et al., 1998), swine HEV ORF2 (Meng et al., 2002), porcine reproductive and respiratory syndrome virus (PRRSV) nucleocapsid (N) and porcine circovirus (PCV) ORF2 (Nawagigil et al., 2002) – and nine synthetic peptides corresponding to truncated avian ORF2 antigenic domains (Guo et al., 2006) were used to coat ELISA plates at 1 µg ml⁻¹ overnight at 4 °C. After blocking and washing the plates, mAbs at various dilutions were added to each well and incubated for 1 h. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM (µ-chain specific; Jackson ImmunoResearch) was used as the secondary antibody.

Flow cytometry analysis. To confirm the IgM isotype, surface IgM profiles of the hybridomas were analysed by flow cytometry on a Becton-Dickinson FACScan. Hybridoma cells (1 × 10⁶) were stained with fluorescein isothiocyanate (FITC)-labelled AffiniPure Monovalent Fab fragment goat anti-mouse IgM (µ-chain specific; Jackson ImmunoResearch) in accordance with a standard protocol provided by the Flow Cytometry Facility at Iowa State University, USA. Normalized mean fluorescence intensities were calculated using CellQuest software (Becton-Dickinson).

Purification and biotinylation of mAbs. mAbs were purified from culture supernatants using anti-mouse IgM (µ-chain specific) agarose affinity columns (Sigma). The purified mAbs were dialysed against 0.01 M PBS (pH 7.2) and subsequently concentrated to 2 mg ml⁻¹ using a Minicon B15 concentrator (Millipore) for biotinylation using EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in accordance with the manufacturer’s protocol.

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 (Zhou & Afshar, 1999), except that the avian HEV ORF2 capsid protein was used as the coating antigen and the residual binding of biotinylated mAbs (B-mAbs) was detected using HRP-conjugated streptavidin (Jackson ImmunoResearch) and a tetramethylbenzidine substrate system (KPL). B-mAbs were used at a dilution that gave an ELISA A⁴₅₀ value of approximately 1.0 (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 inhibition was calculated using the formula: 100 × [1 – (A⁴₅₀ of B-mAb and mAb/ A⁴₅₀ of B-mAb)]. A B-mAb was considered to be inhibited if its binding decreased by 40% or more.

Indirect immunofluorescence assay (IFA). An IFA was used to test further 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 cells, a chicken liver cell line that supports avian HEV replication (Huang et al., 2005). mAbs were used at 10 µg ml⁻¹ and FITC-labelled anti-mouse IgM (KPL) was used at a 1:50 dilution. The remaining procedures were performed as reported previously (Huang et al., 2005).

Binding of mAbs to avian HEV virus. A modified immunocapture RT-PCR method (Li et al., 2001) was used to assess the virus binding ability of the mAbs. Briefly, 100, 200 and 400 ng mAbs or normal mouse IgM (κ-light chain) (Sigma) were coated on ELISA plates (Nunc). Serum samples containing 10 genome equivalents (GE) ml⁻¹ avian HEV from viraemic chickens were added (100 µl per well) and incubated at room temperature for 1 h and then at 4 °C for 12 h. After washing six times, 100 µl viral RNA extraction buffer from an RNAgent Total RNA Isolation system (Promega) was added directly to each well. The RNA was then extracted and reverse transcribed into cDNA with avian myeloblastosis reverse transcriptase (Promega). A nested RT-PCR (Sun et al., 2004) was used to detect the 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 product of 221 bp was confirmed by DNA sequencing. Experiments were repeated twice.
Neutralization assay. Fifteen anti-avian HEV-negative chickens were divided into five groups with three chickens in each group. Avian HEV (1000 GE) from a 10% faecal stock (Guo et al., 2007b) was incubated with 1 mg of each of the four mAbs or normal mouse IgM and the mixtures were incubated at room temperature for 1 h and at 4 °C overnight. Each mixture was subsequently injected into each chicken via the intravenous route. This amount of antibody was used because our preliminary data showed that 1 mg purified anti-avian HEV chicken IgY could completely neutralize 1000 GE avian HEV (H. Guo and others, unpublished results). Sera and faecal swabs were collected before inoculation and weekly thereafter for 5 weeks. Viraemia and faecal virus shedding were detected by a sensitive nested RT-PCR, as described previously (Sun et al., 2004).

Cloning and expression of avian HEV ORF2 fragment proteins. Anti-HEV neutralization epitopes are most likely located in the C-terminal part of the ORF2 capsid protein (Schofield et al., 2003; Zhang et al., 2005). To map the epitopes recognized by anti-avian HEV ORF2 mAbs, four C-terminally truncated avian HEV ORF2 fragments, designated avian HEV ORF2-1 to -4 were constructed (Fig. 1)) using recombinant plasmids containing 804 bp of the avian HEV ORF2 gene (Haqshenas et al., 2002) as the template. To amplify these four fragments, four downstream primers (Avian HEV ORF2-1L: 5'-GGGGATATGGCAGTTGTTGGGCAAAGAACCT-3'; Avian HEV ORF2-2L: 5'-GGGGATATGGCTAGAATACCCACGGCCCTGGG-3'; Avian HEV ORF2-3L: 5'-GGGGATATCATCCACTGTTGCTCCGGAGGA-3'; Avian HEV ORF2-4L: 5'-GGGGATATGGCAGTTGTTGGGCAAAAGAACCT-3') were designed using Oligo 6.0 software (MBI). An EcoRI restriction site (underlined) was engineered into the 5' end of these primers to facilitate subsequent cloning steps. The forward primer containing a BamHI site used for PCR amplification of the 804 bp avian HEV ORF2 (Haqshenas et al., 2002) was used with all four downstream primers. Proofreading Pfu DNA polymerase (Stratagene) was used for all PCR amplifications. The PCR conditions were performed as follows: one cycle at 95°C for 5 min; 35 cycles at 95 °C for 45 s, 51 °C for 45 s and 72 °C for 45 s; one cycle at 72 °C for 10 min. PCR products were digested and purified with a Wizard SV Gel and PCR Clean-Up system (Promega). Purified DNA fragments were cloned into the linearized pRSET-C expression vector (Invitrogen), which encodes a histidine tag and an Xpress epitope at the N terminus of the multiple cloning site. Subsequent expression of these fragments was performed essentially as described previously (Haqshenas et al., 2002), except for avian HEV ORF2-1. For the expression of avian HEV ORF2-1, incubation was performed with a shaking speed of 225 r.p.m. at 25 °C for 6 h after adding 1 mM IPTG. The fusion proteins were partially purified from 100 ml IPTG-induced bacterial culture using BugBuster Protein Extraction reagent (Novagen). Expression of the four truncated avian HEV ORF2 proteins was analysed by Western blotting (Haqshenas et al., 2002) using 1:5000-diluted HRP-conjugated anti-Xpress epitope mAbs (Invitrogen) or 1:1000-diluted polyclonal anti-avian HEV chicken serum.

Mapping of neutralizing epitopes on the avian HEV capsid protein. The epitopes recognized by the mAbs were mapped by Western blotting. Each of the partially purified, truncated avian HEV ORF2 proteins was separated by 15 % SDS-PAGE under non-reducing or reducing conditions and electrophoretically transferred onto a 0.2 μm PVDF membrane (Bio-Rad). The membranes were blocked with 0.01 M PBS/0.05 % Tween 20 containing 2.5 % dried milk overnight with slow shaking and then incubated for 1 h at room temperature with each of the mAbs at a concentration of 4 μg ml⁻¹. Normal mouse serum (diluted 1:100) and mouse anti-avian ORF2 serum (diluted 1:1000) were used as negative and positive controls, respectively. After washing the membranes, HRP-conjugated goat anti-mouse IgM (µ-chain specific; diluted 1:2000) was added and incubated for 1 h. The immune complexes were detected using a DAB Enhanced Liquid Substrate system (Sigma).

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 (data not shown). As IgM antibodies can have polyreactivity (Baccala et al., 1989; Konishi, 1997), the specificity of these mAbs was first tested with different antigens. As shown in Fig. 2, all four 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 1 μg of antibody, the A₄₉₀ values reacting with avian HEV capsid protein were 2.5, 2.3, 2.2 and 1.1 for 5G10, 10A2, 1E11 and 7B2, respectively, indicating that 7B2 had relatively lower binding affinity.

The binding of B-7B2 to avian HEV ORF2 protein was inhibited by homologous 7B2 with 84.8 % inhibition. More complete inhibition was obtained from heterologous 1E11 and 10A2, with inhibition of 95.2 and 96.8 %, respectively (Table 1). Binding of B-1E11 and B-10A2 to avian HEV ORF2 protein was also almost completely inhibited by the homologous mAbs, and they inhibited each other (93.1–97.7 % inhibition). In contrast, 7B2 could only partially inhibit the binding of B-1E11 and B-10A2 to the avian HEV ORF2 protein with 54.4 and 63.1 % inhibition, respectively. These results suggested that 7B2, 1E11 and 10A2 may share the same epitope or overlapping epitopes. There was no significant inhibition (<19 %) between 5G10 and the other three mAbs, indicating that 5G10 probably recognizes a different epitope.

To determine whether the 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
(Huang et al., 2005). 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 transfected cells (Fig. 3a), whilst negative chicken serum did not react with transfected cells (Fig. 3b). At a concentration of 10 μg ml⁻¹, 5G10 showed reproducible binding to the native avian HEV ORF2 capsid protein on the transfected LMH cells (Fig. 3c) and did not react with non-transfected cells (Fig. 3d). None of the other three mAbs reacted with transfected cells (data not shown).

**Fig. 2.** Reactivity of anti-avian HEV ORF2 mAbs with different antigens. ELISA plates were coated with different antigens and reacted with mAbs at various concentrations. The bound mAbs were detected with HRP-conjugated anti-mouse IgM (μ-chain specific). Each point represents the mean ± SEM of the A₄₉₀ value obtained from duplicate ELISA results.

### Table 1. Inhibition of binding of B-mAbs to avian HEV ORF2 capsid protein

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<tr>
<th>B-mAb</th>
<th>Inhibition (%) by unlabelled mAb</th>
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<tr>
<td></td>
<td>7B2</td>
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<tr>
<td>7B2</td>
<td>84.8</td>
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<tr>
<td>1E11</td>
<td>54.4</td>
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<tr>
<td>10A2</td>
<td>63.1</td>
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<td>5G10</td>
<td>8.3</td>
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**Reaction of mAbs with avian HEV particles**

To determine whether the mAbs could capture circulating avian HEV particles, a sensitive nested RT-PCR (Sun et al., 2004) was used to detect the presence of avian HEV captured by the mAbs. As shown in Fig. 4, 1E11, 10A2 and 5G10, when coated at a concentration of 200 or 400 ng per well, captured avian HEV. At a concentration of 100 ng per well, 5G10 was also able to capture avian HEV. Neither 7B2 nor control mouse IgM could capture the virus.

**Neutralization of avian HEV by mAbs**

As there is no cell culture system available that supports 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 established previously (Schofield et al., 2000). Chickens that were inoculated with avian HEV incubated with either normal mouse IgM or 7B2 became infected 1 week after inoculation, shown by faecal virus shedding and viraemia (Table 2). In contrast, chickens inoculated with avian HEV incubated with 1E11, 10A2 or 5G10 did not have detectable avian HEV RNA in their sera or faeces until 3 weeks post-inoculation. These
results suggested that 1E11, 10A2 and 5G10 can at least partially neutralize avian HEV and that infection caused by non-neutralized virus or escaped virus is significantly delayed.

Expression and purification of avian HEV ORF2 fragments

Avian HEV ORF2-1, -2, -3 and -4 fragments were amplified by RT-PCR (Fig. 5a), and subsequently cloned into the pRSET-C expression vector. The recombinant clones were confirmed by DNA sequencing. At 3 h post-induction, the ORF2-2, -3 and -4 proteins with predicted sizes of approximately 23, 18 and 16 kDa, respectively, were expressed and partially purified (Fig. 5b, lanes 3, 2 and 1). Two proteins with approximately sizes of 28 and 16.5 kDa were obtained when bacterial cells transformed with avian HEV ORF2-1 plasmid were induced with IPTG and cultured at 25°C in a shaker at 225 r.p.m. for 6 h (Fig. 5b, lane 4). All fusion proteins were expressed predominantly as inclusion bodies in bacterial cells. Western blot analysis using a mAb 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 blots (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. mAbs 7B2, 1E11 and 10A2 recognized avian HEV ORF2 and ORF2-1 proteins (Fig. 6, lanes 1 and 2), but not the other proteins, suggesting that these mAbs recognized at least one epitope that is located between aa 513 and 570 (Fig. 1 and Fig. 6). In addition to the ORF2 and ORF2-1 proteins, 5G10 also recognized the ORF2-2 protein (Fig. 6d, lane 3), suggesting that the epitope recognized by 5G10 is located between aa 476 and 513. The reaction pattern for each mAb was the 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 1), which implied that 7B2, 1E11 and 10A2, and 5G10 recognize two different epitopes.

DISCUSSION

Avian HEV has been classified in the genus Hepevirus together with the mammalian HEVs (Emerson et al., 2004). Similar antigenic profiles exist between avian HEV and human HEV ORF2 capsid proteins (Haqshenas et al., 2002). Numerous studies have demonstrated that immunization of monkeys with recombinant ORF2 protein of human HEV expressed in Spodoptera frugiperda (Sf9) or bacterial cells confers protection against HEV infection by inducing strong antibody responses (Emerson & Purcell, 2001; Im et al., 2001; Li et al., 2005; Zhang et al., 2001). The Sar-55 HEV-derived recombinant ORF2 protein has been used as a candidate vaccine in phase II/III field trials in Nepal (Shrestha et al., 2007). Our recent data showed that
immunization of chickens with bacterially expressed avian HEV ORF2 protein also induces a protective antibody response (Guo et al., 2007b). The delineation of neutralizing epitopes on avian HEV ORF2 capsid protein could provide further evidence that protection against avian HEV infection by immunization with avian HEV ORF2 protein is probably 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 as, after the second immunization, the mouse anti-avian HEV ORF2 serum IgG titre 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 that 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, as the binding affinity of 7B2 was relatively lower than the other mAbs (Fig. 2). Two antigenic epitopes common to avian and human HEVs mapped previously using synthetic peptides (Guo et al., 2006) were not recognized by these mAbs (Fig. 2). 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 the avian HEV ORF2 protein.

The IFA results using transfected LMH cells suggested that the epitope recognized by 5G10 was intact and exposed, whereas the epitopes recognized by 7B2, 1E11 and 10A2 were either damaged or not exposed on native avian HEV ORF2 proteins. However, following transfection into LMH cells, the RNA transcripts of the avian HEV infectious clone have only a limited level of replication and the replicating virus does not spread from cell to cell (Huang et al., 2005), and thus the IFA is much less sensitive for assessing the binding between mAbs and virus capsid protein. The fact that 1E11 and 10A2 could capture circulating avian HEV particles suggests that the corresponding epitopes on the viral ORF2 protein are exposed and recognized. The virus-capturing ability of these mAbs was specific and affected by their affinity, as normal mouse IgM and the lower-affinity 7B2 did not capture any detectable avian HEV, whereas the higher-affinity mAb 5G10 could capture virus present in a relatively low amount. It has been reported that two anti-Sar-55 HEV chimpanzee mAbs were able to capture and

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**Table 2.** Detection of avian HEV RNA in sera and faecal swabs from chickens inoculated with mixtures of mAb and virus

<table>
<thead>
<tr>
<th>Antibody</th>
<th>No. of positive sera (faecal swabs)/total no. tested* at the following weeks post-infection:</th>
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<td></td>
<td>0</td>
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<tr>
<td>NM IgM†</td>
<td>0 (0)/3</td>
</tr>
<tr>
<td>7B2</td>
<td>0 (0)/3</td>
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<tr>
<td>1E11</td>
<td>0 (0)/3</td>
</tr>
<tr>
<td>10A2</td>
<td>0 (0)/3</td>
</tr>
<tr>
<td>5G10</td>
<td>0 (0)/3</td>
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*Three chickens as a group receiving the same inoculum.
†Normal mouse IgM.

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**Fig. 5.** Expression of avian HEV ORF2 protein fragments. (a) PCR amplification of avian HEV ORF2 fragments. M, DNA marker; lanes 1–4, avian HEV ORF2-4, -3, -2 and -1, with expected sizes of 330, 414, 525 and 696 bp, respectively. (b) SDS-PAGE of partially purified avian HEV ORF2 fragment proteins. M, protein marker; lanes 1–4, avian HEV ORF2-4, -3, -2 and -1, with predicted sizes of 16, 18, 23 and 28 kDa, respectively; lane 5, 32 kDa avian HEV ORF2 protein (Haqshenas et al., 2002). Avian HEV ORF2-1 had two major forms: a predicted 28 kDa protein and a partially degraded 16.5 kDa form. (c) Western blot analysis of expressed proteins detected by HRP-conjugated anti-Xpress epitope mAb. (d) Western blot analysis of the expressed proteins with anti-avian HEV chicken serum. Lanes in (c) and (d) are the same as those shown in (b).
neutralize Sar-55 HEV, whereas other mAbs without virus-capturing ability could not neutralize the virus (Schofield et al., 2003).

The demonstrated virus capturing ability of mAbs 1E11, 10A2 and 5G10 suggested that these mAbs might be able to neutralize virus. We thus performed a neutralization assay. The results showed that 1E11, 10A2 and 5G10 could indeed partially neutralize avian HEV, despite their low efficiency compared with that of anti-avian HEV IgY (data not shown). It is not surprising that, even when used at the same amount as 1E11 and 10A2, 7B2 could not neutralize avian HEV, due to its low affinity and virus-capturing ability.

The epitope defined by 5G10 was between aa 476 and 513 and covered most of the amino acid residues in domain II (aa 461–492) (Guo et al., 2006). However, as peptide 2 derived from the entire domain II did not react with 5G10 (Fig. 2), the actual epitope recognized by 5G10 could be between aa 492 and 513 if it is linear. The epitope recognized by 7B2, 1E11 and 10A2 was between aa 513 and 570 and covered all amino acids of domain III (aa 556–566) (Guo et al., 2006), but, similarly, as peptide 3 representing the entire domain III failed to react with 7B2, 1E11 and 10A2 (Fig. 2), the amino acids of peptide 3 may not be critical components of the epitopes recognized by these three mAbs.

The reported human Sar-55 HEV-neutralizing epitope is one of the major protective epitopes mapped on human HEV and is the essential component of the human HEV recombinant ORF2 protein vaccine (Emerson et al., 2006; Schofield et al., 2000). As 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 (data not shown), these avian HEV neutralization epitopes could also be the major protective epitopes on the 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 (Fig. 2). This is not surprising, as the amino acid sequence identity of the epitope is only approximately 16% among avian, Sar-55 and swine HEVs.

The avian HEV ORF2 protein used for immunization for the production of mAbs was purified from a denatured form (Guo et al., 2006) and thus the mAbs probably recognize linear epitopes. However, the protective epitope on Sar-55 HEV ORF2 protein, which was originally thought to be linear (Schofield et al., 2000), is now considered to be conformational as it is very stable and resistant to heat treatment and reducing agents (Emerson et al., 2006; Schofield et al., 2003; Zhou et al., 2004). Thus, whether the neutralizing epitopes identified on the avian HEV ORF2 are linear or conformational warrants further investigation.

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