Construction of an infectious cDNA clone of genotype 1 avian hepatitis E virus: characterization of its pathogenicity in broiler breeders and demonstration of its utility in studying the role of the hypervariable region in virus replication

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A full-length infectious cDNA clone of the genotype 1 Korean avian hepatitis E virus (avian HEV) (pT11-aHEV-K) was constructed and its infectivity and pathogenicity were investigated in leghorn male hepatoma (LMH) chicken cells and broiler breeders. We demonstrated that capped RNA transcripts from the pT11-aHEV-K clone were translation competent when transfected into LMH cells and infectious when injected intrahepatically into the livers of chickens. Gross and microscopic pathological lesions underpinned the avian HEV infection and helped characterize its pathogenicity in broiler breeder chickens. The avian HEV genome contains a hypervariable region (HVR) in ORF1. To demonstrate the utility of the avian HEV infectious clone, several mutants with various deletions in and beyond the known HVR were derived from the pT11-aHEV-K clone. The HVR-deletion mutants were replication competent in LMH cells, although the deletion mutants extending beyond the known HVR were non-viable. By using the pT11-aHEV-K infectious clone as the backbone, an avian HEV luciferase reporter replicon and HVR-deletion mutant replicons were also generated. The luciferase assay results of the reporter replicon and its mutants support the data obtained from the infectious clone and its derived mutants. To further determine the effect of HVR deletion on virus replication, the capped RNA transcripts from the wild-type pT11-aHEV-K clone and its mutants were injected intrahepatically into chickens. The HVR-deletion mutants that were translation competent in LMH cells displayed in chickens an attenuation phenotype of avian HEV infectivity, suggesting that the avian HEV HVR is important in modulating the virus infectivity and pathogenicity.

INTRODUCTION

Hepatitis E virus (HEV) is the causative agent of endemic and epidemic cases of hepatitis E in many developing countries and sporadic cases of hepatitis E in some industrialized countries (Arankalle et al., 1994; Emerson & Purcell, 2003; Meng, 2010b). The natural route of HEV transmission is faecal–oral via HEV-contaminated water or through contact with infected animals or consumption of infected animal meats (Meng, 2010b; Van der Poel, 2014). Currently, HEV is classified in the family Hepeviridae consisting of a single genus Hepevirus and a floating species avian hepatitis E virus (avian HEV), and the virus is a non-enveloped, positive-sense single-strand RNA virus (Meng et al., 2012). Within the Hepevirus genus, there exist at least four well-characterized and recognized genotypes (John et al., 2014; Meng et al., 2012): genotype 1 and 2 HEVs infect humans while genotype 3 and 4 HEVs are zoonotic and infect humans and several other animal species (Cossaboom et al., 2011; Meng, 2010a; Pavio et al., 2010; Yazaki et al., 2003). More recently, the Hepeviridae Study Group of the International Committee on Taxonomy of Viruses (ICTV) proposed a new classification system that divides the Hepevirus family into two separate genera, Orthohepevirus and Piscihepevirus. Within the Orthohepevirus genus, four distinct species designated Orthohepevirus A to Orthohepevirus D were proposed, and the avian HEV belongs to the Orthohepevirus B species (Smith et al., 2014).
Avian HEV is the causative agent of big liver and spleen disease (BLS) virus from chickens in Australia (Payne et al., 1999), and hepatitis-splenomegaly syndrome (HS syndrome) from chickens in North America (Haqshenas et al., 2001). The morbidity and mortality of BLS or HS syndrome associated with avian HEV infection in the field are low but the drop in egg production can result in substantial losses for the poultry industry (Meng, 2010a). BLS and HS syndrome are often seen in broiler breeders of 30–72 weeks of age in the field (Shivaprasad & Woolcock, 1995; Troxler et al., 2014). Unfortunately, so far, there has been no experimental study to characterize the pathogenicity of avian HEV using broiler breeders, which is the type of chicken that typically develops avian HEV-associated clinical diseases, BLS or HS syndrome, under field conditions (Meng & Shivaprasad, 2013).

Avian HEV is morphologically and genetically related to mammalian HEV, and has been used as a model for the study of human HEV (Billam et al., 2005; Haqshenas et al., 2001; Huang et al., 2005b; Koonin et al., 1992). The genome of avian HEV is approximately 6.6 kb, which is almost 600 bp shorter than that of mammalian HEV. The avian HEV shares common antigenic epitopes with mammalian HEV, even though avian HEV and mammalian HEV share only about 50–60 % nucleotide sequence identity across the entire genome (Guo et al., 2006; Haqshenas et al., 2001, 2002). Based on the comparison of amino acid sequences, the helicase gene, RNA-dependent RNA polymerase (RdRP) gene and capsid gene (ORF2) of avian HEV showed 58–61 %, 47–50 % and 48–49 % sequence identities, respectively, with the corresponding regions of other HEV strains (Haqshenas et al., 2001). Like human HEV, the genome of avian HEV consists of three ORFs: ORF1 encodes a non-structural polyprotein, ORF2 encodes an immunogenic capsid protein, and ORF3 encodes a multifunctional phosphoprotein (Cao & Meng, 2012; Graff et al., 2005; Riddell et al., 2000; Yamada et al., 2009a). It has been reported that the hypervariable region (HVR) in ORF1 overlaps the proline-rich sequence located between the N terminus of the X domain and the C terminus of the putative papain-like protease domain (Meng et al., 1998; Purdy et al., 2012). The HVR varies both in length and in sequence among different HEV strains (Kwon et al., 2012; Smith et al., 2012). The HVR of avian HEV, spanning amino acids 557 to 603 of ORF1, is presumed to be involved in the modulation of HEV replication efficiency (Pudupakam et al., 2011).

At least four different genotypes of avian HEV have been identified from chickens worldwide: genotype 1 from chickens in Australia, genotype 2 from chickens in the USA, genotype 3 from chickens in Europe and China, and genotype 4 from chickens in Hungary and Taiwan (Bányai et al., 2012; Bilic et al., 2009; Haqshenas et al., 2001; Hsu & Tsai, 2014; Huang et al., 2004; Marek et al., 2010; Zhao et al., 2010). The status of avian HEV infection in chickens in Asia is largely unknown since there was only one report of avian HEV detection from China before the virus was identified recently in Korea and Taiwan (Hsu & Tsai, 2014; Kwon et al., 2012; Zhao et al., 2010). The Korean avian HEV belongs to genotype 1. The construction of infectious cDNA clones of HEV provided useful tools to elucidate the structural and functional relationship of HEV genes and facilitate the study of HEV pathogenesis in animal models (Huang et al., 2005a; Okamoto, 2011; Panda et al., 2000; Yamada et al., 2009b). Thus far, only the genotype 2 avian HEV infectious clone is available (Huang et al., 2005b; Kwon et al., 2011). The availability of an infectious cDNA clone of avian HEV (Huang et al., 2005b; Kwon et al., 2011) offered the scientific community an opportunity to use chickens as a useful animal model for HEV. Korean avian HEV belongs to genotype 1, which consists of avian HEV strains only from chickens in Australia (Kwon et al., 2012). Hence, in this study we aimed to construct an infectious cDNA clone using the first Korean strain of avian HEV (HH-F9 strain), characterize its pathogenicity in broiler breeders for the first time, to our knowledge, and assess the roles of the HVR in avian HEV replication and pathogenesis.

RESULTS

Detection of avian HEV-specific antigen in leghorn male hepatoma (LMH) chicken liver cells transfected with capped RNA transcripts from the full-length cDNA clone of Korean avian HEV strain HH-F9

A full-length cDNA clone of the Korean avian HEV strain HH-F9 (pT11-aHEV-K) was constructed by assembling overlapping PCR fragments flanked by unique restriction sites using a stuffer fragment in pGEM-11zf(+) vector. Capped RNAs transcribed from the BamHI-linearized full-length cDNA clone pT11-aHEV-K were used to transfet LMH cells. Avian HEV-specific antigens were detected in transfected LMH cells by an immunofluorescence assay (IFA) using anti-avian HEV convalescent serum, consistent with virus replication in LMH cells, although it cannot be ruled out that the avian HEV antigen could be the result of protein translation from input avian HEV RNA. The positive fluorescent signals were mainly found in the cytoplasm of the avian HEV RNA-transfected LMH cells but not in the mock-transfected cells (Figs 1 and S1, available in the online Supplementary Material).

Capped RNA transcripts of the avian HEV (strain HH-F9) cDNA clone are infectious and produce characteristic liver lesions when injected intrahepatically into the livers of broiler breeder chickens

Four broiler breeder chickens inoculated intrahepatically with capped RNA transcripts of the avian HEV HH-F9 cDNA clone were tested for evidence of avian HEV infection. Avian HEV RNA was detected variably in faeces, and viraemia was also detected variably in serum samples...
in the pT11 K-aHEV group (Table 1). Faecal virus shedding and viraemia were detected from 1 to 2 weeks post-inoculation (wpi) in the pT11-aHEV-K group. All the chickens necropsied at 3 wpi had detectable viral RNA in faeces and sera during the first 3 weeks, and all the chickens necropsied at 6 wpi also had detectable viral RNA in faeces and sera during the course of the 6-week study. All bile and liver samples collected at 3 wpi and 6 wpi were positive for avian HEV RNA (Table 1). Anti-avian HEV antibodies were detected in two out of two chickens necropsied at week 6 of the pT11 K-aHEV group but in neither of the chickens necropsied at week 3. Chickens in the negative control group were seronegative throughout the entire course of the experiment (Table 1).

The liver/body weight ratio and spleen/body weight ratio were higher for the infected group than for the negative control group (Table 1, Fig. 2). In the avian HEV-infected group, microscopic liver lesions including periphlebitis and phlebitis were observed in the portal areas of the liver, which were characterized by severe infiltration of lymphocytes, histiocytes, and a few plasma cells in the vessel walls and perivascular connective tissue. Various sizes of histiocytic foci were observed randomly in the parenchyma, with green–yellow pigments in the lesions (Fig. 2d). Moderate lymphoid hyperplasia was found surrounding the ellipsoid arterioles, and there was a significant increase of the splenic nodules with prominent germinal centre (Fig. 2f).

Deletions of various lengths of the avian HEV HVR reduces the efficiency of avian HEV replication in LMH chicken liver cells

To demonstrate the utility of the genotype 1 avian HEV infectious cDNA clone constructed in this study, we determined the role of the avian HEV HVR in modulating the efficiency of HEV replication using the infectious cDNA clone and its derivatives. An avian HEV luciferase replicon was first constructed and used subsequently to generate three HVR-deletion mutants of the avian HEV luciferase replicon (Fig. 3a). The replication efficiency of the three avian HEV HVR-deletion replicon mutants was analysed in LMH cells. The replication level of the HVRd1-luc mutant was slightly lower than that of the wild-type aHEV-K-luc (P>0.05), whereas the two other avian HEV HVR replicon mutants (HVRd2-luc and HVRd3-luc) replicated at significantly lower levels than the wild-type avian HEV-K-luc did in LMH cells (P<0.05)(Fig. 3b).

Partial deletion of the HVR of avian HEV is dispensable for virus infectivity but complete HVR deletion abolishes virus infectivity in chickens

To further demonstrate the usefulness of this avian HEV infectious cDNA clone in studying virus pathogenicity, we tested the effect of HVR deletions on avian HEV infectivity and pathogenicity in chickens. Capped RNA transcripts from the three HVR-deletion mutants (HVRd1, HVRd2 and HVRd3) and the wild-type avian HEV infectious clone (pT11-aHEV-K) were first transfected into LMH chicken liver cells. On day 6 post-transfection, positive intracytoplasmic fluorescent signals of avian HEV-specific antigens were detected in the LMH cells transfected with RNAs from the wild-type avian HEV as well as the mutant with partial HVR deletion (HVRd1) (Figs 3c and S2). The anti-avian HEV sera likely bind to the viral capsid protein, which is the only known structural protein of HEV. Detection of avian HEV antigen in LMH cells transfected with capped RNAs from the partial HVR-deletion mutant suggested that the partial HVR deletion did not abolish the ability of avian HEV protein translation in LMH cells. However, evidence of avian HEV protein translation, suggestive of virus replication, was not observed in cells transfected with capped RNAs from the other two mutants with longer HVR deletions (HVRd2 and HVRd3) or in the mock-transfected cells (Figs 3c and S2).

Detection of avian HEV-specific antigen in LMH cells transfected with RNA transcripts from wild-type and
Table 1. Detection of avian HEV RNA in faeces, serum, bile and liver, seroconversion to IgG avian HEV antibodies, and liver (spleen)/body weight ratio in broiler breeder chickens inoculated intrahepatically with capped RNA transcripts from the avian HEV HH-F9 cDNA clone (pT11-aHEV-K)

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<th>Group</th>
<th>Chicken ID</th>
<th>Detection of avian HEV RNA and avian HEV antibodies (in parentheses) from each individual chicken at 0–6 wpi</th>
<th>Liver/body weight ratio*</th>
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<td>pT11-aHEV-K</td>
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<td>PBS (negative control)</td>
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F, faeces; S, serum; B, bile; L, liver.  
(−), negative avian HEV antibodies in sera; (+), positive avian HEV antibodies in sera.  
*Liver (spleen) to body weight ratio was calculated by [liver (spleen) weight]/(body weight) × 1000.  
†Necropsied at 3 wpi.  
‡Necropsied at 6 wpi.
Fig. 3. (a) Schematic diagram showing the HVR in ORF1 of Korean avian HEV (aa 557 to 603) and its derived HVR-deletion mutants (HVRd1, HVRd2 and HVRd3), and the avian HEV luciferase replicon and its derived HVR-deletion mutant replicons (HVRd1-luc, HVRd2-luc and HVRd3-luc), along with putative functional domains: MT, methyltransferase; P, papain-like cysteine protease; HEL, helicase. The amino acid sequence of each HVR-deletion mutant is aligned to show the relative positions of the amino acid deletions: mutants HVRd1 (aa 570 to 591), HVRd2 (aa 557 to 607) and HVRd3 (aa 550 to 610). The deletions of the HVR were generated by fusion PCR. The deleted amino acids are shown as a dashed line, and the relative deleted amino acid positions are indicated for each mutant. (b) Luciferase activities in cells transfected with capped RNA transcripts from the parental wild-type avian HEV luciferase reporter replicon and its HVR-deletion mutant replicons. The luciferase activity in LMH cells, which reflects the replication levels of avian HEV RNAs, was measured by determining the firefly luciferase activity at 5 days post-transfection, and normalization for transfection efficiency was performed by using *Renilla* luciferase activity measured at the same time. The value determined with the parental wild-type genotype 1 avian HEV replicon (aHEV-K-luc) was set as 100% and used as a reference to normalize the replication of the other mutant replicons. A replicon carrying an inactivating mutation (GAA) in RdRp served as a negative control. Data are expressed as the mean ± SD of the results of three separate experiments. Asterisks (**) indicate statistical differences compared with the parental wild-type replicon. RLU, relative light units. (c) IFA of LMH chicken liver cells following transfection with capped RNA transcripts from a wild-type avian HEV infectious clone pT11-aHEV-K and its derived HVR-deletion mutants (HVRd1, HVRd2, and HVRd3). Negative control (mock) was transfected with PBS buffer. DAPI staining reveals the nuclei of the cells in blue, FITC staining reveals the avian HEV protein in green, and DAPI+FITC staining represents the merged images. Bars, 4 μm.
the negative control chickens remained seronegative throughout the experiment. Faecal virus shedding and viraemia were detected from 1 to 4 wpi in the positive control wild-type avian HEV pT11-aHEV-K group (Table 2). In the positive control group, four out of the five chickens had detectable viral RNA in faeces or sera, and in the HVRd1-challenged group, only one out of the five chickens had detectable viral RNA in sera during the course of the six-week study. However, none of the 5 chickens in the group inoculated with RNAs from the complete HVR deletion mutant (HVRd2) and none of the chickens in the HVRd1-challenged group, only one out of the five chickens had detectable viral RNA in faeces or sera, and the chickens remained seronegative throughout the study (Table 2). The results demonstrated that avian HEV infectivity tolerates partial deletions in the HVR, although deletion of the complete HVR abolishes avian HEV infectivity in chickens.

Microscopically, the hepatic lesions in the positive control wild-type avian HEV pT11-aHEV-K group were characterized by mild to severe mononuclear cell infiltrations, predominantly lymphocytes, which were generally distributed around the vessels, but often in the midzonal areas in the liver (Fig. 4). Mild hyperplasia of the periartrial lymphatic sheath was noted in the spleen of the chickens in the positive control group. The histological lesion scores of the livers from the chickens in the HVRd1-challenged group and the HVRd2-challenged group decreased markedly, compared with those in the positive control group (P<0.001) (Fig. 4).

**DISCUSSION**

Avian HEV has been identified from mostly broiler breeders and layers in many countries (Bilic et al., 2009; Shivaprasad & Woolcock, 1995; Troxler et al., 2014). The pathogenicity of avian HEV remains largely uncharacterized. There have been a few experimental animal pathogenicity studies done using younger chickens, but not broiler breeders (Billam et al., 2005, 2009; Kwon et al., 2011), which is the type of chicken typically having BLS and HS syndrome in the field. In this study, we successfully constructed an infectious cDNA clone of the genotype 1 Korean avian HEV and characterized its infectivity and pathogenicity for the first time, to our knowledge, in broiler breeders. Additionally, we further demonstrated the utility of the avian HEV infectious clone by constructing various HVR-deletion mutants and luciferase reporter replicon mutants to delineate the functional and structural relationship of the HEV HVR region.

We demonstrated the production of avian HEV-specific antigen in LMH chicken liver cells transfected with the capped RNA transcripts from the pT11-aHEV-K clone, consistent with virus replication. The fluorescent signals were detected in the cytoplasm of the transfected LMH cells, similarly to previous studies (Kwon et al., 2011). We subsequently tested the infectivity of the pT11-aHEV-K clone in broiler breeders by a percutaneous intrahepatic inoculation procedure. The efficiency of the intrahepatic inoculation procedure was verified in a previous study (Huang et al., 2005b) and proved to be an efficient

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<th>Group</th>
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<th>Detection of avian HEV RNA (faecal/serum) from each individual chicken at 0–6 wpi</th>
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procedure again in this study as demonstrated by the development of an active avian HEV infection in the inoculated chickens. Faecal virus shedding and viraemia were detected variably from 1 to 2 wpi in chickens inoculated with RNA transcripts from the pT11-aHEV-K clone, which is consistent with previous studies (Billam et al., 2005, 2009; Huang et al., 2005b; Kwon et al., 2011; Meng et al., 1998). In the pT11-aHEV-K clone group, only the two chickens necropsied at 6 wpi were seroconverted to avian HEV antibodies. This is not surprising, since it typically takes at least 3 weeks for seroconversion to occur under experimental conditions (Billam et al., 2009; Huang et al., 2005b), meaning that the other two chickens in the pT11-aHEV-K group would likely have seroconverted as well if they had not been necropsied at 3 wpi.

In the previous avian HEV infection and pathogenicity studies with young chickens, gross lesions were typically not conspicuous. However, in this study in which we used broiler breeder chickens, the enlargement of liver and spleen was reproduced in infected chickens. The characteristic microscopic lesions of avian HEV infection such as lymphocytic and heterophilic periphlebitis in the liver and lymphoid hyperplasia in the spleen were also observed in the broiler breeder chickens infected with pT11-aHEV-K, which is consistent with previous studies (Billam et al., 2005, 2009; Kwon et al., 2011). Our results obtained in this study using broiler breeder chickens showed more typical gross and microscopic lesions of avian HEV than previous studies with young chickens. The initial objective of the study was to test the infectivity of the genotype 1 Korean avian HEV cDNA clone using broiler breeder chickens and, therefore, the small number of broiler breeder chickens used in this pilot study was insufficient for statistical analysis, but nevertheless the distinguishable characteristic gross lesions were clearly visualized in the infected broiler breeders. A future study with a large number of broiler breeder chickens is warranted to fully characterize the disease and lesions associated with avian HEV infection.

To demonstrate the usefulness of the genotype 1 avian HEV infectious cDNA clone constructed in this study, we
used the infectious clone as the backbone and generated three HVR-deletion mutants and tested the effect of various length HVR deletions on virus infectivity in chickens. We showed that the two mutants (HVRd2 and HVRd3) with HVR deletions beyond the known HVR region (Δ557–603) failed to give rise to detectable levels of viral protein upon transfection into cells and lacked infectivity in vivo. It has been shown previously that the HVR deletion mutants (Δ557–603, Δ566–595, Δ573–587) are all replication competent in LMH cells, suggesting that avian HEV tolerates the deletion of HVR (Pudupakam et al., 2011). In a previous study, the HVR deletion mutants replicated in cells, but the degree of luciferase reporter gene expression was reduced in proportion to the length of HVR deletions (Pudupakam et al., 2011). Consequently, the role of HVR was thought as modulating viral replication efficiency. In this present study, our HVRd1 mutant (Δ570–591) was not affected in its replication efficiency, even though it had a larger HVR deletion than the mutant Avm3-luc (Δ573–587) reported by Pudupakam et al. (2011). The luciferase activity value measured in the HVRd1-luc mutant was almost the same as that of the wild-type replicon statistically. It has been demonstrated that the PxxP motif that binds to the SH3 domain may be involved in enhancing the replication efficiency or infectivity (Bliska, 1996; Macdonald et al., 2005; Pudupakam et al., 2011). A distinctive characteristic of the HVRd1 mutant is the number of proline-rich (PxxP) motifs. The Korean avian HEV strains have four PxxP motifs in the HVR, whereas other avian HEV strains contain only one or two PxxP motifs (Kwon et al., 2012). The HVR-deletion mutant Avm3-luc reported by Pudupakam et al. (2011) had only one PxxP motif in the deleted HVR region, and thus the deletion resulted in not having a PxxP motif at all in the mutant Avm3-luc. On the other hand, the HVRd1 mutant from this present study still contained two PxxP motifs, since only two of four PxxP motifs were in the deleted HVR region. Therefore, this may explain the difference in the results observed between the Avm3-luc mutant from the previous study and the HVRd1 mutant in this present study, since the remaining two PxxP motifs may be sufficient to carry out their presumed function in modulating viral replication efficiency.

Interestingly, the infectivity and pathogenicity of mutant HVRd1, which exhibited efficient virus protein translation competency in vitro, was reduced in vivo when inoculated into chickens. There may exist several possible explanations: firstly, the PxxP motif exerts influence on the virus infectivity. The effect of deletion of two PxxP motifs might be negligible for virus protein translation and replication in vitro, but in the animal host a plethora of other factors such as host immune responses might attenuate the virus such as antagonizing or evading the host immune response (Pudupakam et al., 2011). Based on the analysis of the HVR sequences, it appears that the HVR has multiple predicted functions. In addition to contributing to modulation of virus replication by protein–protein interactions, the HVR also contains sites that bind to various ligands and a wide array of other putative functional sites as well (Purdy et al., 2012).

In conclusion, we report here on the successful construction of an infectious cDNA clone of the genotype 1 Korean avian HEV and have demonstrated the infectivity of its RNA transcripts in broiler breeders for the first time, to our knowledge. We also reproduced the characteristic gross and microscopic pathological lesions in broiler breeder chickens. Additionally, we demonstrated that the genotype 1 Korean avian HEV infectious cDNA clone is very useful in studying HEV replication and pathogenicity. By using HVR-deletion mutant and luciferase reporter replicons, we demonstrated that the HVR plays a role in HEV replication efficiency in vitro and in vivo, and that the PxxP motif within the HVR region may play a pivotal role in modulating virus replication efficiency.

METHODS

Virus and cells. The virus used for the construction of the infectious cDNA clone of Korean avian HEV HH-F9 in the study originated from faecal samples of 60-week-old broiler breeders in Korea (Kwon et al., 2012). The LMH chicken liver cell line (CRL-2117) was purchased from ATCC, and the cells were maintained at 37 °C with 5% CO₂ in Waymouth’s MB752/1 medium (Invitrogen) containing 10% FBS.

Amplification of the full-length cDNA of Korean avian HEV HH-F9 strain by reverse transcription-polymerase chain reaction (RT-PCR). Viral RNA was extracted from the avian HEV strain HH-F9 virus stock and used for cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen) with avian HEV HH-F9-specific reverse primers (Table S1). To amplify the complete genome of avian HEV HH-F9 for the purpose of constructing the infectious cDNA clone, four overlapping fragments (Fig. S3) covering the full-length viral genome flanked by unique restriction enzyme sites were amplified by PCR using four sets of primers which were designed based on the sequence of avian HEV strain HH-F9 (Table S1) essentially as described previously (Kwon et al., 2011). Fragment 1 representing the 5’ end of the viral genome was amplified with primers Salf/T7F1 and AflII1646R1. The forward primer Salf/T7F1 contained, in the 5’ to 3’ direction, an engineered Salf site, the T7 core promoter sequence, and the 5’ end of the avian HEV HH-F9 sequence (Table S1, fragment 1). The reverse primer, T18BamHIR, that was used to amplify fragment 4 representing the 3’ end of the viral genome contained 18 adenosine nucleotides and a BamHI restriction site at the end of the avian HEV HH-F9 genome sequence. A stuffer fragment covering all the unique restriction sites (Salf, AflII, Agel, Kpnl and BamHI) was produced by PCR with two overlapping synthetic oligonucleotides. All RT-PCR-amplified fragments were purified from agarose gels, and cloned in the TOPO TA cloning vector (Invitrogen). Individual cDNA clones were sequenced, and the clones with verified sequence of avian HEV strain HH-F9 were used for the assembly of the final full-length cDNA clone.

Construction of a full-length cDNA clone of avian HEV strain HH-F9. The plasmid vector pGEM-11zf(+) (Promega) was first modified by replacing the fragment between the Salf and BamHI sites...
with the synthetic stuffer fragment produced in this study as described above. Each of the independent cDNA fragments (Fig. S3, f1 through f4) was excised from the respective recombinant vectors, gel-purified, and ligated into pGEM-11zf(+) vector after digestion of pGEM-11zf(+) vector with the same restriction enzymes. After each ligation step, each recombinant pGEM-11zf(+) plasmid was transformed into One Shot TOP10 competent Escherichia coli cells (Invitrogen) and grown overnight at 37 °C in the presence of ampicillin. The assembled full-length cDNA clone was designated pT11-aHEV-K.

**In vitro transcription and transfection.** The full-length cDNA clone pT11-aHEV-K was linearized by digestion with BamHI, and subsequently purified by phenol/chloroform extraction and ethanol precipitation. The linearized plasmid DNA was used to transcribe capped RNA transcripts with T7 polymerase using the mMESSAGE mACHINE T7 ULTRA kit (Ambion). Briefly, each reaction was performed in a 20 μl reaction mixture containing 1 μg linearized cDNA template, 2 μl 10 × reaction buffer, 10 μl 2 × NTP/Cap, 2 μl enzyme mix and an additional 1 μl 30 mM GTP stock. The mixtures were incubated at 37 °C for 2 h. The quality of the RNA transcripts was tested by loading 0.5 μl of the reaction mixture on a 1.0 % agarose gel. The capped RNA transcripts from the full-length cDNA clone of avian HEV strain HH-F9 were used directly for the in vitro transcription of LMH chicken liver cells. As a positive control, capped RNA transcripts from the infectious cDNA clone, pT7-aHEV-VA (Kwon et al., 2011), were also produced. For the in vivo study in broiler breeders, each in vitro RNA transcription reaction was performed in a 100 μl reaction to generate capped RNA transcripts from the BamHI-linearized pT11-aHEV-K cDNA clone. The capped RNA transcripts were diluted 1:4 with cold (stored at 4 °C) RNase- and proteinase-free PBS buffer (pH 7.4), frozen on dry ice, and used for inoculation of each chicken the next day.

**Immunofluorescence assay.** To test the ability of viral protein translation and replication of the pT11-aHEV-K clone in vitro, LMH chicken liver cells were transfected with the capped RNA transcripts of the pT11-aHEV-K clone and pT7-aHEV-VA clone in a 12-well plate by using a Lipofectamine LTX kit (Invitrogen). On day 5 post-transfection, the cells were fixed and stained by an IFA. Briefly, cells of the pT11-aHEV-K clone and pT7-aHEV-VA clone in a 12-well plate by using a Lipofectamine LTX kit (Invitrogen). The stained cells were viewed under a fluorescence microscope (Olympus).

**Evaluation of the infectivity of pT11-aHEV-K clone in broiler breeder chickens.** The animal experiment in this study was approved by the Animal Care and Use Committee of Kangwon National University. Six 37-week-old broiler breeder chickens (Ross-PS) that tested negative for avian HEV RNA and antibodies were purchased from Samhwa Breeding (Hongseong, Korea) and randomly divided into two groups of four and two chickens each. Each chicken was housed in an individual isolator. Percutaneous intrahepatic inoculation of capped RNA transcripts of avian HEV was performed essentially as described previously (Huang et al., 2005b). The capped RNA transcripts were injected directly into two different sites of the right lobe of the liver. Four chickens in the pT11-aHEV-K group were each inoculated intrahepatically with 400 μl capped RNA transcripts from the pT11-aHEV-K clone. Two chickens in the negative control group were each inoculated intrahepatically with 400 μl sterile PBS (pH 7.4). Faecal samples obtained by swabbing the cloaca and colorectum, and serum samples were collected prior to inoculation and weekly thereafter from each chicken. Weekly serum and faecal samples were tested for avian HEV RNA by RT-PCR and weekly serum samples were tested by an avian HEV-specific ELISA with a purified recombinant avian HEV capsid antigen for anti-HEV antibodies as described previously (Sun et al., 2004). One step RT-PCR (Qiagen) and PCR premix (Invitrogen) using primer pairs specific for the Korean avian HEV strain HH-F9 were used to detect avian HEV RNA (Table S1). Two chickens in the pT11-aHEV-K group and one chicken in the negative control group were euthanized and necropsied at 3 wpi. The remaining chickens from each group were necropsied at 6 wpi. During each necropsy, each chicken and its liver and spleen were weighed, and the liver/body and spleen/body weight ratios were calculated. bile and liver samples were also collected from each chicken during each necropsy and tested for avian HEV RNA by RT-PCR. The liver tissues were fixed in 10 % neutral buffered formalin, processed routinely for paraffin embedding, sectioned at 3–5 μm thickness, and then stained with haematoxylin and eosin (H&E). The stained liver tissue sections were examined under a light microscope (Olympus) for evidence of histological lesions.

**Generation of an avian HEV luciferase reporter replicon.** To demonstrate the utility of the avian HEV infectious clone, we constructed an avian HEV luciferase reporter replicon (aHEV-K-luc) using the avian HEV infectious cDNA clone expressing firefly luciferase. The firefly luciferase gene (Promega) was inserted in-frame with the start codon of the avian HEV ORF2 to replace nt 4713 to 6362 of the avian HEV infectious clone pT11-aHEV-K (Fig. 3a). The final fusion PCR product was substituted for the SbfI-to-FseI region in the avian HEV infectious clone. As a negative control, a null replication mutant of the avian HEV replicon, AvGAA- luc, was also constructed by mutating the conserved GDD motif of the avian HEV RdRp to GAA by using a QuickChange lightning site-directed mutagenesis kit (Agilent Technologies) with primers AvGAA-F and AvGAA-R (Table S1).

**Construction of HVR-deletion mutants of avian HEV as well as HVR-deletion mutants of the avian HEV luciferase reporter replicon.** To demonstrate the usefulness of the avian HEV infectious cDNA clone in studying the replication of the virus, we constructed three HVR-deletion mutants with various lengths using fusion PCR and the pT11-aHEV-K infectious cDNA clone as the backbone (Figs 3a and S3). Amino acid residues 570 to 591, 557 to 607, and 550 to 610, corresponding to nt 1693 to 1845, 1732 to 1797, and 1672 to 1854, were deleted to produce the three HVR-deletion mutants HVRd1, HVRd2 and HVRd3, respectively. The two fragments used for fusion PCR were first amplified with the primer sets SalIT7F1(A)/HVRD1B and HVRD1C/ORF1-SbfI(D) for the mutant HVRd1, SalIT7F1(A)/HVRD2B and HVRD2C/ORF1-SbfI(D) for the mutant HVRd2, and SalIT7F1(A)/HVRD3B and HVRD3C/ORF1-SbfI(D) for the mutant HVRd3 (Table S1). The PCR products amplified from each mutant were then used in the fusion PCR with primer set SalIT7F1(A)/ORF1-SbfI(D) (Table S1). The fusion product was purified, digested with SalI and SbfI, and ligated into the backbone of pT11-aHEV-K with the SalI–SbfI region deleted to produce the HVR-deletion mutants.

For the construction of avian HEV luciferase replicon mutants, the fusion products of in-frame amino acid deletions (A570–591, A557–607 and A550–610) in ORF1 were engineered into the backbone of the aHEV-K-luc replicon to produce the three HVR-deletion mutant replicons HVRd1-luc, HVRd2-luc and HVRd3-luc, respectively (Fig. 3a).

**Transfection of avian HEV HVR-deletion mutants in vitro.** To determine the viral protein translation and replication of the HVR-deletion mutants in vitro, the LMH chicken liver cells were transfected

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with capped RNA transcripts from pT11-aHEV-K and its derived HVR-deletion mutants (HVRd1-luc, HVRd2-luc, HVRd3-luc) using a Lipofectamine LTX kit (Invitrogen) as described above. At 5 days post-transfection, LMH cells were trypsized and replated on 24-well plates. On day 6, the LMH cells were rinsed with PBS, fixed with a solution containing 70% acetone and 30% ethanol, and stained by FITC and DAPI as described above.

**Luciferase assay to measure the effect of HVR deletions on virus replication using the avian HEV luciferase replicon reporter system.** The HVR-deletion mutant luciferase replicon aHEV-K-luc and its derived mutants (AvGAA-luc, HVRd1-luc, HVRd2-luc and HVRd3-luc) were linearized with BamHI and purified by phenol/chloroform extraction and ethanol precipitation. Capped RNA transcripts were synthesized with the mMESSAGE mMACHINE T7 ULTRA kit (Ambion) from the mutant and wild-type replicons. Each transcription mixture was cooled on ice and used for the transfection of LMH cells. LMH chicken liver cells were transfected at approximately 85% confluence with RNA transcripts in a 12-well plate by using a Lipofectamine LTX kit (Invitrogen). For the dual-luciferase assay, pRL-TK Renilla luciferase reporter construct (Promega) was co-transfected to each luciferase assay sample (aHEV-K-luc, AvGAA-luc, HVRd1-luc, HVRd2-luc and HVRd3-luc) for normalization and as an internal control. LMH cells at 5 days post-transfection were washed with PBS and lysed. The cell lysates were centrifuged briefly and 20 μl of supernatant was used for luciferase assays with a dual-luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Luciferase activities were measured using a Tecan Safire2 microplate reader (Promega).

**Evaluation of the infectivity and pathogenicity of pT11-aHEV-K and its HVR-deletion mutants in specific-pathogen-free (SPF) chickens.** The pT11-aHEV-K infectious cDNA clone and its derived HVR-deletion mutants HVRd1 and HVRd2 were linearized by digestion with BamHI and purified by phenol/chloroform extraction. Each in vitro transcription reaction was performed in a 50 μl reaction as described above for the production of capped full-length RNA transcripts. The capped RNA transcripts from each cDNA clone were subsequently diluted 1:9 with cold RNase-, DNase- and proteinase-free PBS buffer, frozen on dry ice, and used for intrahepatic inoculation of chickens on the next day.

For the percutaneous intrahepatic inoculation procedure of capped RNA transcripts into the livers of chickens, 20 three-week-old SPF chickens (White Leghorn, Namduk SPF, Korea) that were negative for avian HEV were divided into four groups of five chickens per group. The RNA transcripts were injected into two different sites of the liver, with approximately 250 μl (approx. 75 μg) per injection site (Huang et al., 2005b). The five chickens in the pT11-aHEV-K group were each injected with a total of 500 μl of RNA transcripts from the wild-type avian HEV infectious cDNA clone (pT11-aHEV-K) as positive controls. The five chickens in the HVRd1 group were each injected intrahepatically with RNA transcripts from a partial HVR-deletion mutant clone (HVRd1). The five chickens in the HVRd2 group were each injected intrahepatically with RNA transcripts from the complete HVR-deletion mutant clone (HVRd2). The five chickens in the mock negative control group were each injected similarly with PBS buffer as negative controls. Faecal swabs and sera were collected from each chicken prior to inoculation and weekly thereafter, and were tested by RT-PCR for avian HEV RNA (Table S1). Weekly serum samples were also tested by ELISA for seroconversion to avian HEV antibodies as described above. All chickens were euthanized using carbon dioxide and necropsied at 6 wpi. During necropsy, each chicken and its liver were weighed and the liver/body weight ratio was calculated. Liver tissues were fixed in 10% neutral-buffered formalin, processed routinely for paraffin embedding, sectioned at 3 μm thickness and then stained with H&E. The stained slides were examined under a light microscope (BX41, Olympus) for histopathological liver lesions. Inflammatory foci were counted in the liver section under the light microscope. The severity of hepatic inflammation was recorded and assigned scores according to the scoring system described previously (0, no lesions; 1, <5 foci of inflammation; 2, 5–8 foci; 3, 9–15; 4, >15 foci) (Billam et al., 2009).

**Statistical analyses.** One-way ANOVA followed by Tukey’s post hoc test was used for multiple comparisons of the experimental groups based on the level of their replication in LMH cells. Significant differences were defined as those having a P value of <0.05. The histological liver lesion scores were compared between the groups using the Kruskal–Wallis test followed by Dunnet’s method for multiple comparisons against the K group and the numbers of inflammatory foci were compared between the groups using ANOVA followed by Dunnet’s test for multiple comparisons against the K group. The valid data were analysed statistically and presented as mean ± SEM.

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