Co-infection with avian hepatitis E virus and avian leukosis virus subgroup J as the cause of an outbreak of hepatitis and liver hemorrhagic syndromes in a brown layer chicken flock in China

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ABSTRACT Hens of a commercial Hy-line brown layer flock in China exhibited increased mortality and decreased egg production at 47 wk of age. From 47 to 57 wk, average weekly mortality increased from 0.11 to 3.0%, and egg production decreased from 10 to 30%, with a peak mortality rate (3.0%) observed at 54 wk of age. Necropsy of 11 birds demonstrated tissue damage that included hepatitis, liver hemorrhage, rupture, and/or enlarged livers. Microscopic liver lesions exhibited hepatocytic necrosis, lymphocytic periphlebitis, and myeloid leukosis. While no bacteria were recovered from liver and spleen samples, avian hepatitis E virus (**HEV**) RNA was detected in all 11 tested hens by nested reverse transcription-polymerase chain reaction. Of these, subgroup J avian leukosis virus (**ALV-J**) proviral DNA was detected in 5 hens by PCR. Alignments of partial ORF2 gene sequences obtained here demonstrated shared identity (76 to 97%) with corresponding sequences of other known avian HEV isolates. *Env* sequences of ALV-J isolates obtained here shared 50.1 to 55% identity with other ALV subgroups and 91.8 to 95.5% identity with other known ALV-J isolates. Phylogenetic tree analysis of selected sequences obtained here grouped an avian HEV sequence with genotype 3 HEV and assigned an ALV-J sequence to a branch separate from known ALV-J subgroups. Immunohistochemical results confirmed the presence of avian HEV and ALV-J in livers. Therefore, these results suggest that avian HEV and ALV-J co-infection caused the outbreak of hepatitis and liver hemorrhagic syndrome observed in the layer hen flock analyzed in this study.

Key words: avian hepatitis E virus, subgroup J of avian leukosis virus, ORF2 gene, *env* gene, co-infection

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INTRODUCTION

Avian hepatitis E virus (**HEV**) is the main causative agent of big liver and spleen (**BLS**) disease, hepatitis–splenomegaly (**HS**) syndrome, and hepatic rupture hemorrhage (**HRH**) syndrome. The clinical signs of these diseases include increased mortality (1–5%), decreased egg production (10–40%), accumulated abdominal blood, liver hemorrhage, and enlarged livers and spleens in broiler breeder and laying hens (Payne et al., 1999; Haqshenas et al., 2001; Su et al., 2018). However, there is accumulating evidence that this virus may not be sufficient to cause disease (Sun et al., 2004). Notably, avian HEV has been detected in healthy chickens; while under experimental conditions, clinical disease has not been consistently reproduced in specific-pathogen-free (**SPF**) chickens inoculated with avian HEV (Billam et al., 2005). Therefore, it has been speculated that factors in addition to avian HEV are required before BLS, HS syndrome, or HRH syndrome symptoms are observed in broiler breeder and laying hens. Currently, avian HEV infection of chicken flocks is generally diagnosed using 2 assays: an indirect enzyme-linked immunosorbent assay (**iELISA**) to test for the presence of antibodies and reverse transcription-polymerase chain reaction (**RT-PCR**) to detect viral RNA in liver, fecal, and bile samples (Huang et al., 2002; Sun et al., 2004; Zhao et al., 2013). Recently, several nested RT-PCR methods

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have been developed for diagnosing avian HEV infection in flocks (Peralta et al., 2009; Zhao et al., 2010). Among these methods, RT-PCR assays developed by Huang et al. have been widely used (Huang et al., 2002).

Avian leukosis viruses (**ALV**) have been subclassified into 6 subgroups, designated A-E and J, which are based on their host range, as well as on viral envelope interference and cross-neutralization patterns (Payne et al., 1991; Bai et al., 1995). The subgroup J of avian leukosis virus (**ALV-J**) was first isolated from commercial meat-type chickens in the United Kingdom in the late 1980s (Payne et al., 1992). Chickens infected with ALV-J usually exhibit tumors development, depressed immunity, growth retardation, liver hemorrhage, enlarged liver and spleen, and increased mortality that is most apparent in broiler breeder hens, with some clinical signs shared with BLS disease, HS syndrome, and HRH syndrome (Arshad et al., 1997). In China, ALV-J infection has emerged in meat-type chicken and layer and breeder hen flocks (Xu et al., 2004; Chen et al., 2005; Sun et al., 2007). However, co-infection of ALV-J and other chicken viral agents, such as Marek's disease virus, reticuloendotheliosis virus, or chicken infectious anemia virus, have been frequently reported (Jiang et al., 2005; Jin et al., 2010; Qin et al., 2010). For diagnosis of ALV-J infection in chicken flocks, virus isolation, ELISA, and PCR assays are widely used (Gao et al., 2014; Sun et al., 2014; Dai et al., 2015).

In this study, an investigation was performed to identify the causative agent of disease observed in a layer hen flock exhibiting increased mortality and decreased egg production. For diagnosis of avian HEV and ALV-J infection, ELISA and nested RT-PCR, PCR, and immunohistochemical assays were conducted and demonstrated that natural co-infection with avian HEV and ALV-J had occurred in the layer flock.

MATERIALS AND METHODS

Farm Description and Clinical Observations

In October 2017, a disease outbreak with elevated mortality and decreased egg production was observed in a chicken flock on a farm located in the city of Xi'an (34°17′N and 108°93′E) within Shaanxi Province, China. The flock consisted of Hy-line brown layer hens previously were vaccinated with live vaccines against Newcastle disease (LaSota), infectious bronchitis (4/91, H120 and Ma5), Marek's disease (CVI988+Fc126), infectious bursal disease (strain 2,512), and *Mycoplasma gallisepticum* and with inactivated vaccines against avian influenza virus (H5, H9) and avian rhinotracheitis.

The farm was 48,000 square-feet in size and was a commercial Hy-line laying hen facility, with an average house capacity of 8,000 birds and a total of 6 houses. Each house was managed based on all-in, all-out routine practices and received replacement pullet flocks from a single source. In one of 6 houses, increased mortality emerged in 47-week-old hens. During the following 11 wk, weekly mortality increased from an average of 0.11 to 3.0% in the flock and peaked (3.0%) at 54 wk of age (Figure 1). Egg production, which began at 18 wk in the house, decreased by 10 to 30% during the 11-wk period for hens of 47 to 57 wk of age (Figure 1). The other houses on the farm functioned normally, with mortality below 0.08% and egg production rates at approximately 93% (data not shown).

Sample Collection, Histopathology, and Bacteriology

A total of 480 serum samples were collected from chickens in the 6 houses (80 per house) and tested to detect antiviral antibodies. The blood samples are collected through wing vein, incubated for 30 min in

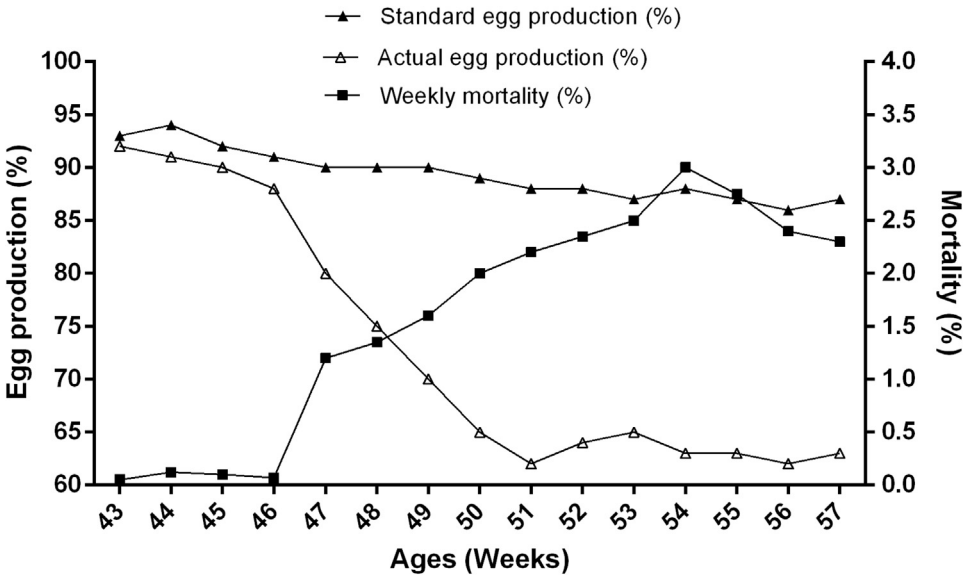

Figure 1. Egg production and mortality rates from one house (flock) exhibiting decreased egg production and increased mortality of chickens 47 to 57 wk of age.

37°C and then stew overnight in 4°C. Serum samples were harvested by centrifugation at 3,000 *g* for 10 min. In addition, eleven 54-week-old chickens exhibiting serious clinical symptoms or death were selected randomly from the house with emerging disease. Serum samples and chicken specimens were submitted to the laboratory for testing and diagnosis. Livers were collected from the 11 selected chickens and part of each liver was fixed in 10% neutral buffered formalin, dehydrated by passing through a graded series of ethanol solutions, embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin and eosin before examination via light microscopy. In addition, liver sections were immunohistochemically tested to detect the presence of avian HEV and ALV-J as described below. Meanwhile, liver, spleen, bile, and stool samples were also collected from the selected diseased chickens and stored at -80°C for viral RNA and DNA detection. Liver and spleen samples for bacteriological analysis were taken from a limited number of birds. Bacteriological analysis was performed as described (Landman and Van Eck, 2017).

RNA Extraction and Nested RT-PCR Assay

All collected tissues and fecal samples (of approximately 1 g each) were minced and diluted 1:10 in phosphate-buffered saline (**PBS**, pH 7.2), frozen and thawed 3 times then centrifuged at 3,000 × *g* for 10 min to obtain supernatants. Bile samples were directly diluted 1:10 in PBS and centrifuged at 3,000 × *g* for 10 min to obtain supernatants. Next, 200 μL of each sample supernatant was used for total RNA extraction using an EasyPure Viral RNA Kit (TransGen Biotech, Ltd., Beijing, China). Extracted RNA was subsequently used for detection of the partial ORF2 gene of avian HEV.

Detection of avian HEV RNA was performed using a nested PCR procedure previously described by Sun et al. (Sun et al., 2004). Briefly, an external primer set consisting of ORF2/F-1/SD : 5′-TCGCCT(C)GGTAAT(C)ACA(T)AATGC-3′ and ORF2/R-1/SD : 5′-GCGTTC(G)CCG(C)ACAGGT(C)CGGCC-3′ and an internal primer set consisting of ORF2/F-2/SD : 5′-ACA(T)AATGCT(C)AGGGTCACCCG-3′ and ORF2/R-2/SD : 5′-ATGTACTGA(G)CCA(G)CTG(C)GCCGC-3′ were used, whereas the primer ORF2/R-1/SD was also used as reverse transcription primer. Reverse transcription was performed using M-MLV reverse transcriptase (TaKaRa, Dalian, China) based on the manufacturer's instructions under the following conditions: 42°C for 60 min and 85°C for 15 min. First-round PCR was performed using 5 μL cDNA as template with added 2 × rTaq PCR Master Mixture (**TaKaRa**) and thermal cycling using the following conditions: 95°C for 10 min, 39 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 45 s, with a final incubation at 72°C for 10 min. The second-round PCR used 1 μL of first-round PCR product as template and 2 × rTaq PCR Master Mixture. Thermal cycling conditions consisted of 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final incubation at 72°C for 10 min. The expected size of second-round PCR products was approximately 278 bp.

DNA Extraction and PCR Assay

For the ALV-J, PCR using RNA as a template is applied to identify the virus infection. Nevertheless, the virus could synthesize double-stranded DNA and integrate DNA into the host chromosome (Smith et al., 2018). The operation of extracting DNA is simpler than that of RNA, and most references reported that the proviral genes of ALV were used to PCR amplification and followed sequencing the PCR products for viral detection (Meng et al., 2016; Smith et al., 2018). And then, the DNA was extracted for ALV-J detection. Liver and stool samples were minced according to the aforementioned method for DNA extraction using a viral DNA extraction kit (Biomiga, San Diego, CA). Next, extracted DNA was used to detect ALV proviral DNA via PCR. Universal primers for detecting the ALV *env* gene were ALV (all)-F: CGAGAGTGGCTCGCGAGATGG and ALV (all)-R: ACACTACATTTCCCCCTCCCTAT. PCR was performed using the same 2 × rTaq PCR Master Mixture as described for nested PCR with 5 μL DNA as template. Thermal cycling conditions were as follows: 95°C for 5 min, 39 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final incubation at 72°C for 10 min. The expected size of PCR products was approximately 1,016 bp.

Sequencing and Phylogenetic Analysis

Second-round PCR products for detection of the avian HEV partial ORF2 gene and PCR products for detection of ALV *env* were size-separated electrophoretically using 0.8% agarose gels then purified using an EasyPure Gel Extraction Kit (TransGen Biotech, Ltd.). Each PCR product obtained for avian HEV and ALV was sequenced using an ABI 3730 Genetic Analyser (JinSiTe Biotech Co., Nanjing, China). For gene identification, nucleotide sequences were analyzed by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST).

For sequence analysis of the amplified partial ORF2-encoding gene of avian HEV, primer binding sites were excluded, and sequences of a 242-bp region were subjected to multiple alignments using the MegAlign program within the Lasergene software package (DNASTAR Inc., Madison, WI). In addition, according to the previous studies, the phylogenetic tree constructed based on the 242 bp is same as the one based on the complete genomes (Sun et al., 2004; Bilic et al., 2009). So, using MEGA5.1 software, a phylogenetic tree based on the 242 bp sequences was constructed of sequences obtained here and other known avian HEV strains obtained from GenBank. GenBank accession numbers of avian HEV sequences used for sequence comparisons and tree construction included KP221201,

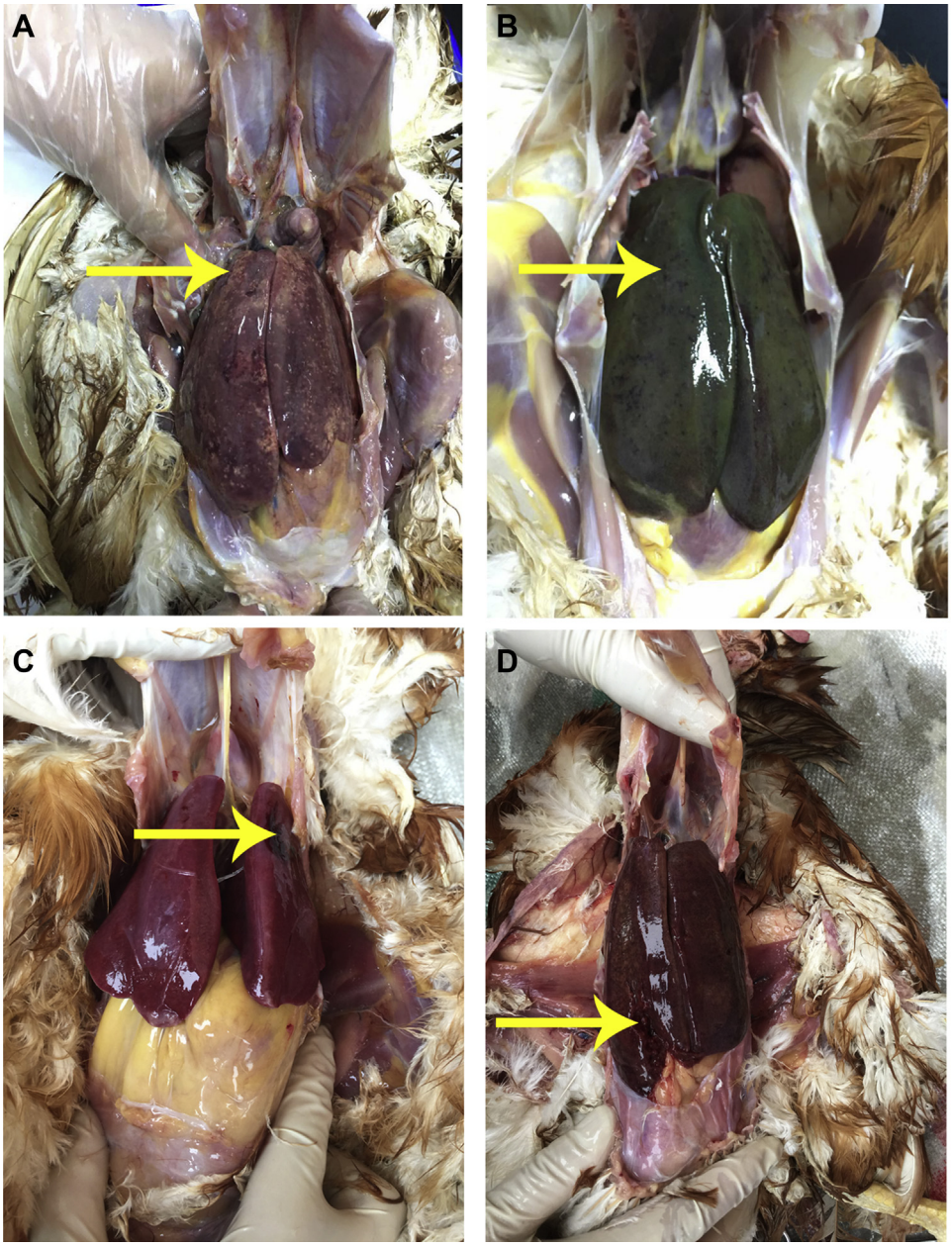

Figure 2. Enlarged liver, hepatitis syndrome, and liver hemorrhage in affected layer hens. (A) Yellow hepatic necrosis visible in livers. (B) Dark livers. (C) Diffuse liver hemorrhage. (D) Hepatic rupture hemorrhage.

FM872318, AM943646, FM872312, MK050107, AY870825, AY870819, KF511797, JN997392, FM872311, AM943647, FM872313, EU919189, AY535004, EF206691, AY870831, MG737712, and KX589065. Out of which, some isolates are the standard ones, and others are the representative ones. However, these isolates have been used to alignment and construct trees in a previous study (Sun et al., 2004). For the *env* gene of ALV, amplified DNA sequences (976 bp) were also compared with each other and with sequences of various ALV strains obtained from GenBank. Next, a phylogenetic tree was also constructed for ALV sequences using the aforementioned software. GenBank accession numbers of ALV sequences were AY027920, Z46390, FJ216405, GU982310, HM235669, DQ115805, AY897228, AY897232, AY897222, AY897221, AY897223, AY897219, KY490695, M37980, AY013303, J02342, M14902, and M33292. Also, these isolates have been used to construct trees in some previous studies (Meng et al., 2018; Wang et al., 2018).

Detection of Antibodies from Serum Samples

The 480 collected serum samples were used to detect antiavian HEV IgG antibodies using an iELISA based on the method described by Zhao et al. (Zhao et al., 2013). For detection of anti-ALV-J IgG antibodies, a

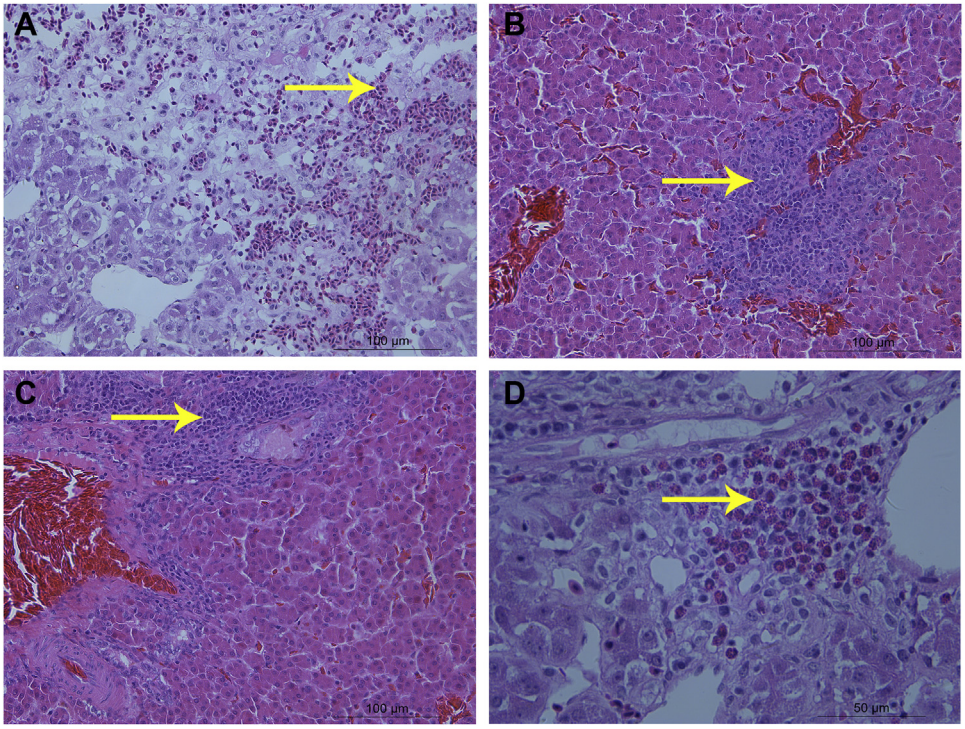

Figure 3. Microscopic lesions of livers of layer hens from affected house. (A) Image of hemorrhage in liver; Bar = 100 um. (B) Lymphocytes and focal hyperplasia in liver lobule; Bar = 100 um. (C) Lymphocytic infiltration of hepatic portal area; Bar = 100 um. (D) Myelocyte-like tumor cells filled with eosinophilic particles; Bar = 50 um. Tissues were stained with hematoxylin and eosin.

commercial ELISA test kit (FlockChek® Avian Leucosis Virus Antibody Test Kit Subgroup J, IDEXX, FlockChek®) was used as per the manufacturer's instructions. Based on the iELISA assay, a sample result was considered positive when the optical density values at 450 nm (OD_{450nm}) of testing serum samples were greater than 0.368 (Zhao et al., 2013). For detection of anti-ALV-J antibodies, a sample was considered positive if the result obtained using the formula (OD_{650nm} value of test sample—OD_{650nm} value of negative control)/(OD_{650nm} value of positive control—OD_{650nm} value of negative control) (S/P) was greater than 0.6 (based on the instructions of the commercial ELISA kit).

Immunohistochemistry

To detect and localize avian HEV and ALV-J viral proteins within liver tissues, immunohistochemical (**IHC**) assays were performed. Tissue sections were fixed in formalin then embedded in paraffin blocks. Blocks were sliced into thin sections using a microtome, then sections were affixed to poly-L-lysine-coated glass slides. Next, slides were de-paraffinized using Histo-choice and rehydrated in graded ethanol baths. After flooding slides with 3% H_2O_2 in methanol, the slides underwent antigen retrieval via heating in a microwave oven in citrate buffer at pH 6.0. After washing in PBS, slides were treated with 5% BSA for 10 min to block nonspecific binding. Next, slides were separately incubated in antiavian HEV ORF2 (Dong et al., 2011) or ALV-J monoclonal antibodies (Qin et al., 2002) for 1 h at 37°C. After washing with PBS, slides were incubated with goat anti-mouse IgG (H+L)-HRP conjugate for 1 h at 37°C then washed. **DAB** (3,3′-diaminobenzidine tetrahydrochloride) was added as a component of the DAB Enhanced Liquid Substrate Source. Slides were counterstained with hematoxylin for 1 min, washed in water for 5 min, and dehydrated then mounted using balsam.

RESULTS

Gross Lesions and Histological Examination

Of 11 chickens from the affected house, 7 exhibited severely enlarged livers with numerous yellow necrotic liver lesions (Figure 2A). The other 4 chickens exhibited dark livers with hepatomegaly (Figure 2B). Notably, all 11 chickens exhibited diffuse liver hemorrhages (Figure 2C) with liver rupture observed in 3 chickens (Figure 2D). Microscopic examination of livers demonstrated the presence of hemorrhages and severe hepatic degeneration and necrosis (Figure 3A), lymphocytes with focal hyperplasia within liver lobules (Figure 3B) and focal aggregation of lymphocytes around central

Table 1. Percent identities (%) between the 32 avian HEV isolates in the study and the known different genotypes avian HEV strains in the GenBank based on 242 bases of ORF2 fragments.

Isolates	Each other	Different genotypes of avian HEV (% identity)			
		Genotype 1	Genotype 2	Genotype 3	Genotype 4
CHN-SX-aHEV-1 to 32	97–100	82–84	76–80	95–97	81–86

Abbreviation: HEV, hepatitis E virus.

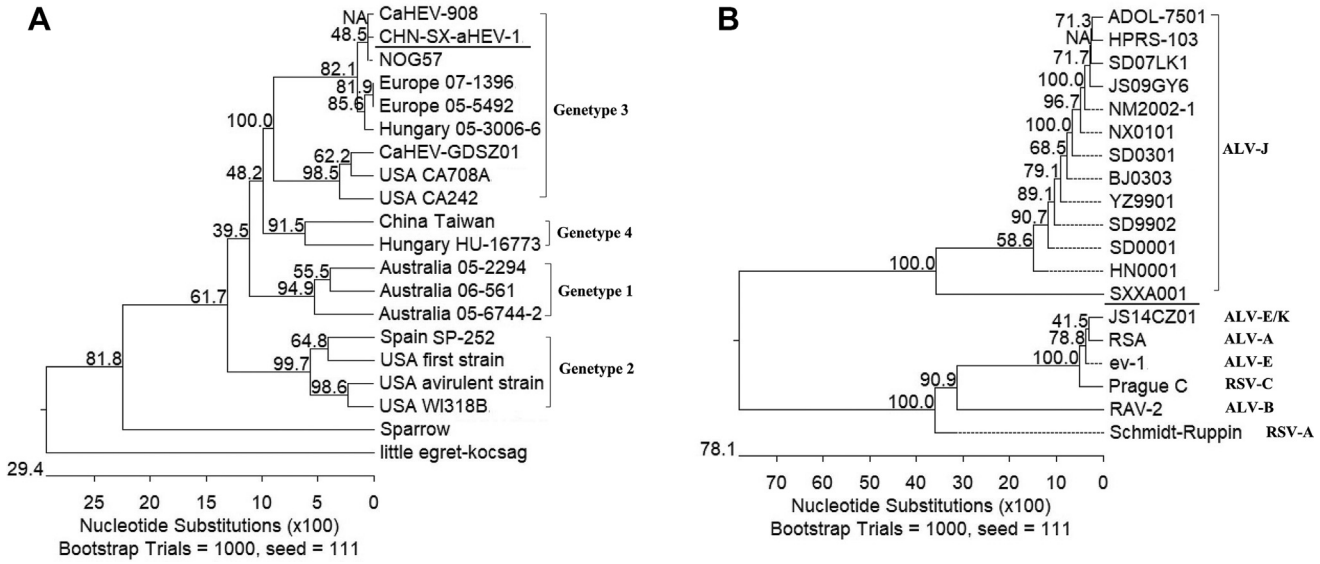

Figure 4. Phylogenetic trees constructed via the Neighbor-Joining method using Lasergene software (DNASTAR Inc., Madison, WI) based on nucleotide sequence alignments. (A) Tree based on partial ORF2 gene of avian HEV (242 bp). (B) Tree based on env gene of ALV-J (964 bp). Number of nucleotide substitutions per 111 residues are given; number of bootstrap trials is set to 1,000. Sequences from the present study are underlined. Abbreviations: ALV-J, J avian leukosis virus; HEV, hepatitis E virus.

veins (Figure 3C). Such microscopic lesions were similar to pathological changes typically caused by avian HEV infection of chickens. In addition, lymphoid tumor cells and small numbers of myeloid cells containing cytoplasmic eosinophilic particles were also observed in liver sections (Figure 3D), which are typical pathological changes observed in birds with ALV-J infection. Of the 11 liver sections, all showed typical pathological changes characteristic of avian HEV infection. Of these, 7 also exhibited changes that are typically observed during ALV-J infection.

Bacteriology

No bacterial agent was isolated from any liver or spleen using standard aerobic and anaerobic bacteria isolation protocols.

Virology

Avian HEV RNA was detected in all 11 tested chickens, with 11 liver, 11 bile, 7 stool, and 3 spleen samples testing positive for the partial avian HEV ORF2 gene sequence using nested RT-PCR. In addition, 5 chickens (5 liver and 3 stool samples) that were also avian HEV RNA positive were positive for the ALV env gene via PCR detection. BLAST results showed that all avian HEV RNA-positive sample sequences shared approximately 80–99% identity with known avian HEV strain sequences obtained from GenBank, while sequences of ALV positive samples shared 90–95% identity with GenBank ALV-J sequences. Reticuloendotheliosis virus and clinical Marek's disease virus were not detected in these samples (data not shown). When considered together, these results suggest that the tested flock was co-infected with avian HEV and ALV-J.

Sequence Comparisons and Phylogenetic Tree Construction

The 32 sequences of the 242-bp partial avian HEV ORF2 gene PCR products obtained from all positive samples, designated CHN-SX-aHEV-1 to 32, shared 97 to 100% nucleotide sequence identity with each other and 76 to 97% identity with sequences of other GenBank reference strains of avian HEV (Table 1). These sequences shared the highest degree of identity (95–97%) with avian HEV genotype 3 isolates from the United States, Europe, and China (Table 1). Based on sequence comparisons, CHN-SX-aHEV-1 was selected, submitted to the GenBank (No. MK829812), and used to construct a phylogenetic tree with other known avian HEV strains obtained from GenBank. The results showed that CHN-SX-aHEV-1 clustered with avian HEV strains from the United States, Europe, and China and belonged to genotype 3 avian HEV (Figure 4A).

The *env* gene ALV sequences of 964-bp PCR products of positive samples, designated SXXA001 to SXXA008, shared 96 to 100% with each other, 91.8 to 95.5% identity with other reference ALV-J sequences and 50.1 to 55% identity with other subgroup ALV sequences (Table 2). A phylogenetic tree based on the *env* gene showed that SXXA001 clustered within the ALV-J group in a separate branch (Figure 4B). The sequence of SXXA001 has been submitted to the GenBank, and the accession No. is MK829813.

Detection of Antibodies Against Avian HEV and ALV-J in Sera

The 480 sera from the flock were tested for antiavian HEV antibodies using iELISA and for anti-ALV-J

Table 2. Percent identities (%) between the 8 ALV-J isolates in the study and the known different subgroups of ALV-J strains in the GenBank based on complete *env* genes.

		Different subgroups of ALV (% identity)						
Isolates	Each other	A	B	C	D	E	J	E/K
SXXA001 to 008	96–100	52–53	47–48	52–53	50.1–51	53–55	91.8–95.5	52–55

Abbreviation: ALV-J, J avian leukosis virus.

antibodies using a commercial ELISA kit. For detection of antiavian HEV antibodies in the 80 sera collected from the flock exhibiting decreased egg production, 62 samples (77.5%) were positive (Figure 5A). However, of the other 400 sera, no samples tested positive (OD_{450nm} values of all 400 samples were <0.2, data not shown). Regarding anti-ALV-J antibodies, for the 80 sera from the affected house, 43 (53.75%) were positive, and all 43 were also positive for antiavian HEV antibodies (Figure 5B). For the other 400 sera, all tested negative for anti-ALV-J antibodies (S/*P* values of all 400 samples were <0.2, data not shown).

Immunohistochemical Detection

Testing to detect avian HEV and ALV-J in liver tissues of 11 selected chickens via IHC assay demonstrated that all 11 liver tissues exhibited positive cytoplasmic staining signals for avian HEV in hepatocytes (Figure 6A), and of these, 5 were also positive for cytoplasmic and nuclear staining of ALV-J (Figure 6B). Therefore, IHC results were in agreement with detection results of nested RT-PCR (avian HEV RNA) and PCR (ALV-J proviral DNA).

DISCUSSION

Both avian HEV and ALV-J infections in chicken flocks have been reported in China (Chen et al., 2005; Zhao et al., 2010). Regarding avian HEV infection, it has been reported that the virus circulates frequently in some chicken flocks in the eastern region of China (Su et al., 2019). Unfortunately, while ALV-J infection now rarely occurs in white-feather broilers, it has gradually returned to local chicken farms in China (Sun et al., 2007). In a previous study, it had been documented that anti-avian HEV and anti-ALV-J antibodies were detected in the same broiler and layer flocks, triggering

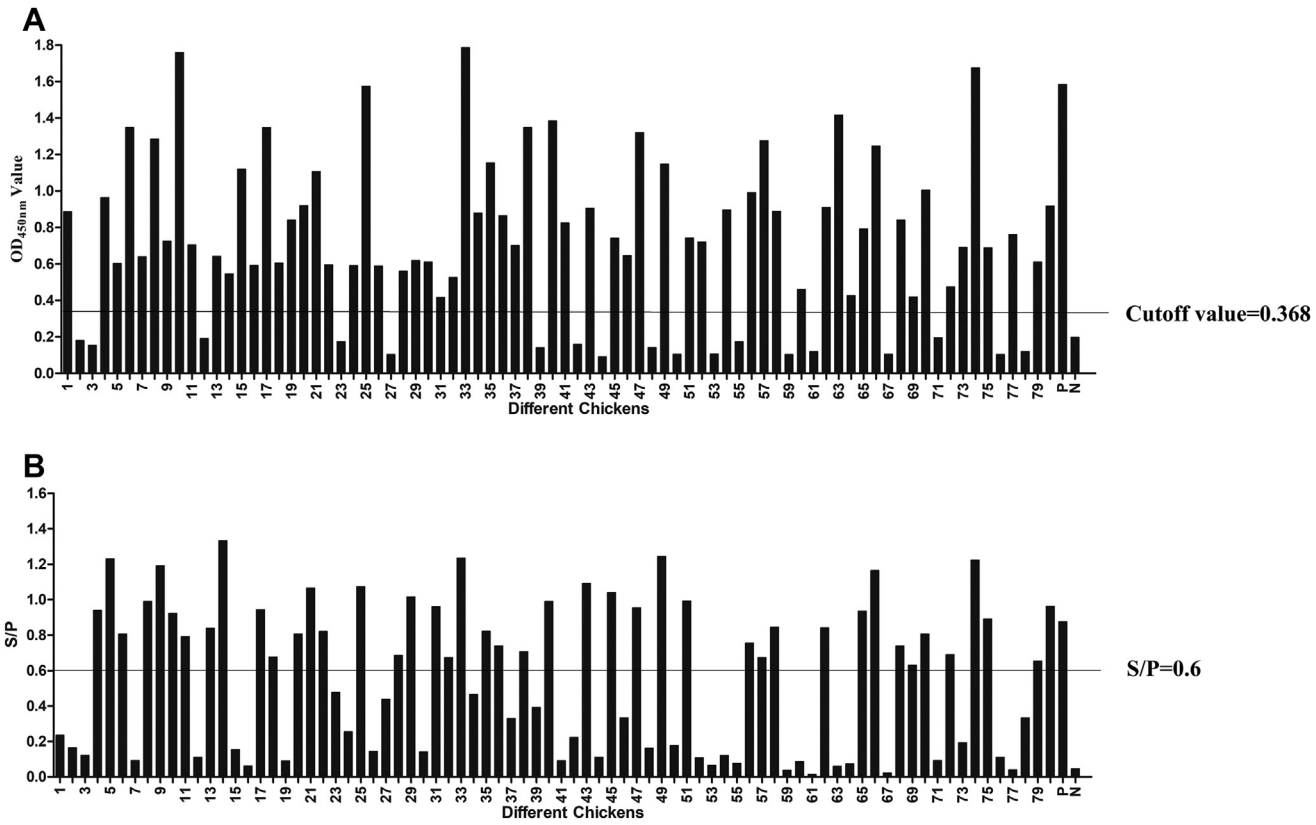

Figure 5. Detection of antibodies against avian HEV and ALV-J in serum samples from chickens from the diseased house. The 2 lines indicate cut-off values of the iELISA for detecting antibodies against avian HEV and the commercial ELISA kit for detecting anti-ALV-J antibodies. (A) The OD_{450nm} value of iELISA results for each serum sample. (B) The S/*P* value of the commercial ELISA kit testing for each serum sample. Abbreviations: ALV-J, J avian leukosis virus; HEV, hepatitis E virus; iELISA, indirect enzyme-linked immunosorbent assay.

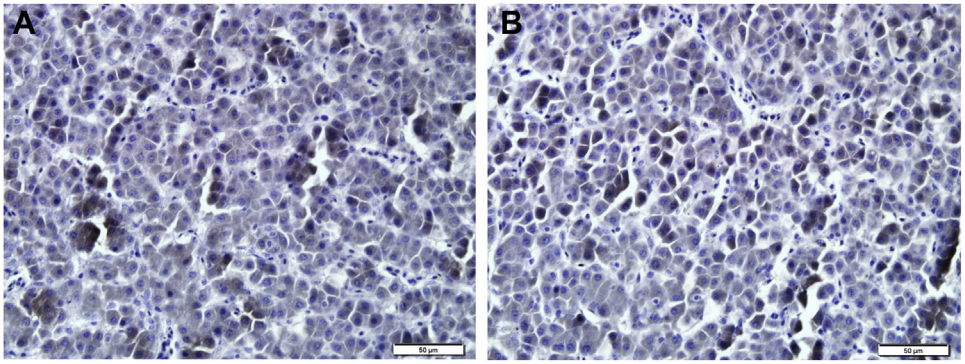

Figure 6. IHC analysis of avian HEV and ALV-J infection of layer hens from the affected house. IHC staining of livers was performed to detect avian HEV (A) or ALV-J (B). (A) Positive staining apparent in cytoplasm and nuclei of liver cells; Bar = 50 um. (B) Positive staining of liver cytoplasm was very pronounced; Bar = 50 um. Abbreviations: ALV-J, J avian leukosis virus; HEV, hepatitis E virus; IHC, immunohistochemical.

speculation that avian HEV and ALV-J may co-infect the same flock (Sun et al., 2016). In the present study, avian HEV RNA and ALV-J proviral DNA could be detected together in individual chickens, with livers of those chickens also exhibiting distinct microscopic lesion types induced by both viruses. These results therefore confirm that both viruses can co-infect the same chicken. Notably, this is the first report demonstrating that avian HEV and ALV-J can naturally co-infect the same chicken.

In previously described field cases, avian HEV RNA was detected in chickens with BLS disease, HS syndrome, HRH syndrome, and fatty liver hemorrhagic syndrome (Payne et al., 1999; Haqshenas et al., 2001; Agunos et al., 2006; Su et al., 2018). However, HEV RNA was also detected in apparently healthy chickens, arousing suspicion that avian HEV may not be the sole causative agent of clinical disease (Sun et al., 2004). Moreover, additional host factors that include stress, hormonal, or immune status, as well as other disease conditions, might also contribute to causation of disease outbreaks (Marlet et al., 2018). In this report, mixed infection with avian HEV and ALV-J was described in a Hy-line layer flock suffering from decreased (10–30%) egg production, increased (1–3%) mortality rate, liver hemorrhage, necrosis, and hepatomegaly. The results suggest that co-infection with avian HEV and ALV-J can cause HRH syndrome in chickens. Nevertheless, additional animal experiments under experimental conditions are needed to confirm the causative agent of HRH syndrome.

Based on phylogenetic tree analysis, avian HEV characterized in the present study belonged to genotype 3, the genotype of most avian HEV isolates identified in China (Zhao et al., 2010; Liu et al., 2017). Recently, a novel avian HEV genotype was characterized in chickens with HRH syndrome (Su et al., 2018). However, that sequence is not yet available from GenBank, preventing comparisons between the avian HEV isolate obtained here and the novel sequence mentioned in the recent HRH study. Regarding ALV-J infection, the disease has been controlled to some degree, because of successful eradication programs implemented in China (Zhou et al., 2019). In fact, there have been only a few reports of chicken infections on local farms in recent years (Sun et al., 2007). Nevertheless, in this study, ALV-J infection was detected in a Hy-line brown layer hen flock, suggesting that the disease may be occurring sporadically in some chicken flocks and on some local chicken farms in China.

Both avian HEV and ALV-J can cause subclinical and persistent infections of chickens, resulting in important economic losses for the poultry industry (Billam et al., 2005; Li et al., 2018). However, to date, no vaccines or drugs are available for prevention and treatment of these 2 diseases (Molaei et al., 2006; Meng, 2010; Zhao et al., 2017; Yu et al., 2019). Indeed, eradication programs implemented since 2010 have prevented ALV-J disease in most chicken flocks in China, with only rare cases reported (Wang et al., 2018). By contrast, no such program to prevent and control avian HEV infection in chicken flocks has yet been implemented but is urgently needed.

This report documents avian HEV and ALV-J co-infection of Hy-line brown layer hen flocks in China, resulting in high mortality, reduced egg production, overt disease, and histologically detectable liver damage. Using experimental detection methods for bacterial and viral detection, we determined that both avian HEV and ALV-J were causes of this outbreak. Further in vitro investigations and animal experiments are necessary to elucidate the pathogenesis of avian HEV and ALV-J co-infection in chickens.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://10.1016/j.psj.2019.10.067.

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