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Letter

Natural co-infection of fowl adenovirus type E-8b and avian hepatitis E virus in parental layer breeders in Hebei, China

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Dear Editor,

Fowl adenovirus (FAdV) and avian hepatitis E virus (aHEV) are the most important causative agents of hepatic injury, representing one of the greatest health concerns in layer chicken flocks (LCFs) in China and causes substantial economic losses. FAdVs are non-enveloped, double-stranded DNA viruses containing a genome of 43–45 kb in size and belong to the genus *Aviadenovirus* in the family of *Adenoviridae* (Hess, 2000). Based on restriction enzyme digestion patterns and serum cross-neutralization tests, FAdV is currently clustered into five species (FAdV-A–FAdV-E) with 12 serotypes (FAdV-1 to -7, FAdV-8a, FAdV-8b, and FAdV-9 to -11). Different serotypes of FAdVs cause different clinical symptoms in poultry flocks. FAdV-1 can induce gizzard erosion in chickens, whereas inclusion body hepatitis (IBH) in chickens is often associated with FAdV-2, -8a, -8b, and -11. FAdV-4 causes hydropericardium hepatitis syndrome and IBH in chickens, and its infections have caused severe economic losses in the global poultry industry (Ye et al., 2016). Avian hepatitis E virus (aHEV) is a major causative agent of big liver and spleen disease (BLS), hepatitis splenomegaly syndrome, and hepatic rupture hemorrhage syndrome (HRHS) in chickens (Sun et al., 2019). Currently, aHEV infection is widespread in chicken flocks in China (Su et al., 2018). Clinical signs of these diseases include increased mortality (1%–5%), decreased egg production (10%–40%), abdominal blood accumulation, liver hemorrhage, and enlarged livers and spleens in broiler breeder and laying hens (Hsu and Tsai, 2014). Avian HEV is a single-stranded positive-sense RNA virus with a genome of approximately 6.6 kb in length, consisting of 3 open reading frames (ORFs). ORF2 encodes the capsid protein which contains the major antigenic epitopes of virus that has been proved to be closely associated with the induction of viral infection and immune responses in host cells.

In poultry farms, viral co-infections have occurred increasingly and frequently. Recently, FAdV infections have been described as co-infections involving pathogens such as infectious bursal disease virus (IBDV), avian reoviruses (ARV), Marek's disease virus (MDV), and chicken infectious anemia virus (CIAV) in China (Yang et al., 2016; Meng et al., 2018; Yu et al., 2019; Sun et al., 2020). The increasing viral spread within the poultry sector represents a major concern regarding the economic consequences of co-infection. Therefore, FAdV co-infection with other infectious agents has become a critical issue. Presently, data on co-infection of FAdV and aHEV remain unavailable. Here, we present a study in China on the FAdV and aHEV co-infection associated with severe clinical signs and even death in chickens.

LCFs of a certain Hy-line Brown parental breeder showed increased deaths with a mortality rate of up to 40% in 8000 parental breeding hens at approximately 80 days on a farm located in Handan City in Hebei Province, China. The vital clinical features observed were anorexia, depression, ruffled feathers, and huddling. Autopsy of the dead chickens with 70–75 days old was performed consecutively for 5 days. Postmortem findings revealed typical symptoms of liver enlargement in 140 dead chickens, several chickens had liver rupture, whereas few chickens had splenomegaly (Supplementary Fig. S1). Since aHEV and FAdV represent causative agents of BLS and hepatic HRHS in chickens, we hypothesized that the flock was infected with aHEV or/and FAdV based on the clinical symptoms. As shown in the flow chart, antibody detection, nucleic acid detection, virus isolation and identification, and gene sequence analysis were used to identify the infectious pathogen (Supplementary Fig. S2).

Three-hundred sera samples from the infected chicken flocks were collected for antibody detection, and positive rates were found to be 80% and 16% for FAdV and aHEV, respectively. To further determine the causative agent in this case, an FAdV digoxigenin-labeled probe assay (Hou et al., 2022) and a nested reverse transcription-polymerase chain reaction (RT-nPCR) were performed to detect the nucleic acids of FAdV

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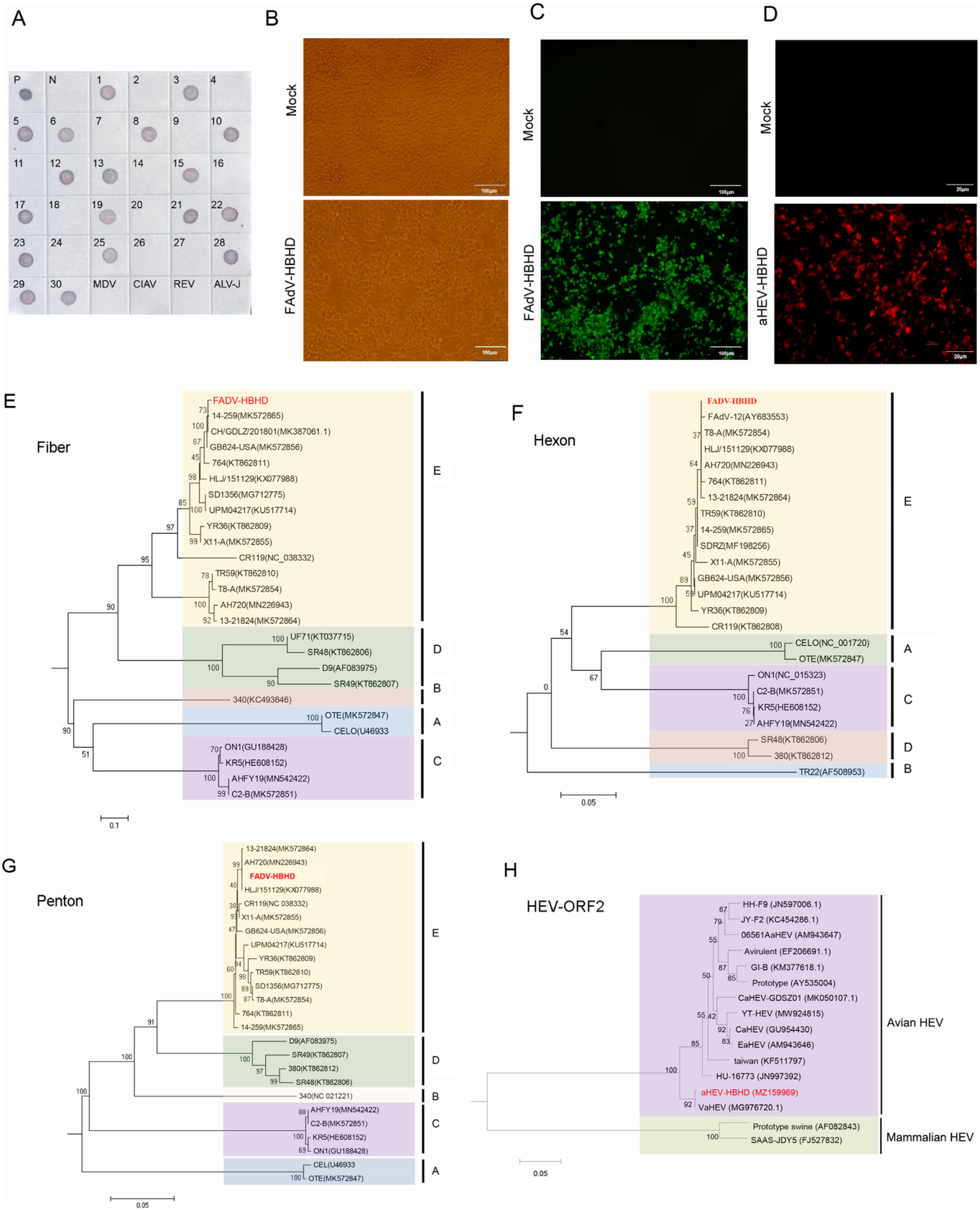

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and aHEV, respectively. DNA from liver samples of 30 randomly selected dead chickens was analyzed using an FAdV digoxigenin-labeled probe assay and it showed 18/30 positive ratio (Fig. 1A). Among the 18 FAdV-positive dead chickens, nine exhibited aHEV infections based on RT-nPCR—a standard method for diagnosing aHEV infections (see Supplementary Materials) (Sun et al., 2004). Therefore, approximately 30% of the dead chickens were co-infected with FAdV and aHEV. Additionally, FAdV was successfully isolated from the liver samples of the dead chicken using a cross-neutralization test with animal hyperimmune sera and limiting dilution in Leghorn male hepatoma (LMH) cells. Typical cytopathic effects (CPE), including rounding, clumping of cells, and detachment from the surface (Hou et al., 2021) were observed on day 5 after liver slurry was inoculated into LMH cells as compared with characteristics of the control (Fig. 1B). The cell culture supernatant was inoculated into new LMH cells after day 5, and an immunofluorescence assay (IFA) was performed using the penton protein-specific monoclonal antibodies against all 12 FAdV serotypes. Typical fluorescence signals were observed within the infected cells using fluorescence microscopy (Fig. 1C). Furthermore, FAdV was successfully isolated after plaque purification and several passages, and named as the FAdV-HBHD strain. Moreover, aHEV was successfully isolated from chicken liver samples using the same method used for FAdV isolation. Since aHEV-infected LMH cells do not exhibit visible CPE, we confirmed the isolation of aHEV via IFA using polyclonal antibodies against the ORF2 of aHEV. Red fluorescence signals were detected in the cytoplasm of infected cells, whereas the mock control failed to exhibit any red fluorescence (Fig. 1D). These results indicated that an aHEV strain was successfully isolated from the LMH cell culture system and thus, was designated as the aHEV-HBHD strain.

To further characterize the serotypes/genotype of FAdV and aHEV circulating in these chicken flocks, full-length sequences of *fiber*, *penton*, and *hexon* genes of FAdV and the *ORF2* gene of aHEV were amplified using gene-specific primers (Supplementary Table S1). Subsequently, the four corresponding fragments were sequenced. Sequence data were deposited in GenBank and are available under accession numbers MW735943, MW735942, MW748221, and MW748221. BLAST comparison analysis using sequences of different reference strains of FAdV showed that the *fiber*, *penton*, and *hexon* genes of the FAdV-HBHD strain and E-8b nucleic acid exhibited the highest similarity at 98.73%, 100%, and 100%, respectively. Phylogenetic analysis, based on *fiber* (Fig. 1E), *hexon* (Fig. 1F) and *penton* (Fig. 1G) genes of FAdV-HBHD, revealed that the new isolate was most closely related to E-8b strain of FAdV. Especially, based on the phylogenetic tree analysis of *fiber* genes (Fig. 1E), it was found that the newly isolated strain had the closest evolutionary relationship with E-8b, with a homology of 93.84%–98.70%. However, it shares 80.65%–85.15% homology with 8a. aHEV has been reported in Shandong, Hebei, and Guangdong provinces in China, where different genotypes with high genetic polymorphism have been identified. In the current study, BLAST comparison analysis using the *ORF2* gene of aHEV-HBHD showed that the aHEV-HBHD strain exhibited the highest similarity at 99.4% with an aHEV field isolate VaHEV (GenBank accession no.MG976720.1), indicating that there may have been a same or similar origin between them. An evolutionary tree based on *ORF2* revealed a close relationship between the two reference strains (Fig. 1H). The similarity was 80.7% between aHEV-HBHD and the aHEV strain isolated from imported white-feather broilers that were recently reported by our laboratory (Zhang et al., 2022), whereas the similarity was 81.3% between aHEV-HBHD and with the CaHEV strain (Genbank accession no. GU954430), the first aHEV strain isolated in China. Hence, our results also highlight the fact that aHEV shows great variation in chickens. The FAdV and aHEV reference strains used for constructing the evolutionary tree are shown in Supplementary Tables S2 and S3.

Then, blood samples were obtained from 60 chicks that hatched from eggs of FAdV-infected layer hens. Both digoxigenin-labeled probe and PCR analyses (data not shown) showed that all these samples were positive for FAdV infection. Therefore, all chicks were infected with FAdV via vertical transmission, additionally proving that FAdV is transmitted vertically (Grgic et al., 2006). Such high vertical transmission rate may be related to the high virulence of FAdV. In contrast, since no aHEV-positive nucleic acid was detected in these commercial laying hens, the evidence was insufficient to confirm the existence of vertical transmission of aHEV in chicken flocks. To our knowledge, there is no complete chain of evidence to prove the vertical transmission of aHEV. Mainly, there are no deterministic literature reports that aHEV has been detected from chicken blood or feces. We also did not detect co-infection of FAdV and aHEV in the 1-day-old commercial chickens, even though 30% of the parental chickens were found to be co-infected with FAdV and aHEV. In the past, chicken mortality caused by FAdV-C4 infection was the main concern in China, while FAdV-8b infection which appears to be less virulent has often been overlooked. The current case reminds us that the concurrence of FAdV-8b and aHEV infections can also be responsible for the high mortality in chickens.

In conclusion, our study describes the co-infection of FAdV-8b and aHEV in parental LCFs in China, and is the first study to report the co-infection to the best of our knowledge. In the current case, although the FAdV-8b and aHEV tended to show low pathogenicity by themselves, their co-infection led to increased mortality, suggesting that viruses exhibiting low pathogenicity by themselves cannot be ignored in a state of co-infection. Thus, continuous monitoring of FAdV and aHEV infection, regular vaccination against FAdV, and control of aHEV in poultry flocks may help mitigate economic losses caused by such co-infections. Additional *in vitro* investigations and animal experiments are essential to elucidate the pathogenesis of FAdV and aHEV co-infection in chickens.

Footnotes

All studies involving animals were conducted according to the animal welfare guidelines of the World Organization for Animal Health. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. This work was supported by the National Key Research and Development Program of China (2022YFD1800602) and the Public welfare project of China Institute of Veterinary Drug Control (GY202104, 202105, GY202012, and GY202008).

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Fig. 1. Co-infection of FAdV type E-8b and aHEV. **A** Detection of FAdV nucleic acid in liver samples using digoxigenin-labeled probes. P: Positive control; N: Negative control; 1–30#: sample. MDV: Marek's disease virus; CIAV: Chicken infectious anemia virus; REV: Reticuloendotheliosis virus; ALV-J: Avian leukosis virus subgroup J. **B** Cytopathic effects of FAdV-HBHD infection. Mock: LMH cells without infection; FAdV-HBHD: LMH cells infected with FAdV on day 5 after inoculation. Scale bar = 100 μm. **C** IFA of LMH cells infected with FAdV-HBHD. Mock: LMH cells without infection as a negative control; FAdV-HBHD: LMH cells infected with FAdV-HBHD detected using IFA with mAb against penton of the FAdV. **D** Fluorescence staining of LMH cells infected with aHEV-HBHD. Mock: LMH cells without infection; aHEV-HBHD: LMH cells infected with aHEV-HBHD detected via IFA with pAb against ORF2 of aHEV. Phylogenetic trees were constructed using the maximum likelihood method based on the nucleotide sequences of *fiber* (**E**), *hexon* (**F**), *penton* gene (**G**), and *ORF2* (**H**) sequences from aHEV. Numbers on the branches represent branch lengths. The isolates FAdV-HBHD and aHEV-HBHD are labeled using red stars. FAdV, fowl adenovirus; aHEV, avian hepatitis E virus. Experimental details are provided in the Supplementary materials.

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