The Diagnosis and Molecular Epidemiology Investigation of Avian Hepatitis E in Shandong Province, China

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Abstract

Background: Avian hepatitis E virus (HEV) is the pathogenic agent of big liver and spleen disease (BLS) and hepatitis-splenomegaly syndrome (HS) in chickens, which has caused economic losses to the poultry industry in China. Eighteen samples of BLS chickens were collected in this study to understand the molecular epidemiology characteristics of avian HEV in Shandong province, China.

Results: Gross and microscopic lesions of clinical samples were observed, then virology detection and genetic analysis of avian HEV were performed. The results showed that there were significant swelling and rupture in the liver, and spleen was enlarged. Microscopic lesions demonstrated that obvious hemorrhage in the liver, with infiltration of heterophilic granulocytes, lymphocytes, and macrophages, the reduction of lymphocytes in the spleen. Eleven out of the 18 samples were positive for HEV, with a positive rate of 61.11%. More importantly, all HEV positive samples were mixed infections. Among them, the mixed infections of avian HEV and chicken infectious anaemia virus (CIAV) and fowl adenovirus (FAdV) were the most common. In addition, the genetic evolution analysis showed that all obtained HEV isolates did not belong to the reported 4 genotypes, and they constituted a novel genotype.

Conclusions: These results of this study further enriched the epidemiological data of avian HEV in Shandong province and proved the genetic diversity of HEV in China, but also uncovered the complicated mixed infections of avian HEV in clinical.

Background

Avian hepatitis E virus (HEV) can cause big liver and spleen disease (BLS) and hepatitis-splenomegaly syndrome (HS) in chickens, characterized by hepatosplenomegaly. Avian HEV mainly infects laying hens and broiler breeders through fecal-oral transmission route [1], resulting in a decrease in laying rate and an increase in mortality [2]. Since first avian HEV strain was isolated and identified in China in 2010 [3], the disease caused by avian HEV has been increasingly prevalent in chickens in recent years, and caused substantial economic losses to chicken industry in China.

Avian HEV is a member of hepevirus and possesses a single-stranded positive-sense RNA genome of approximately 6.6 kb in size, which contains three open reading frames (ORFs) and 3’ and 5’ non-coding regions. Of which, capsid protein encoded by ORF2 is highly conserved and has been extensively used for viral genotyping and genetic evolution analysis of HEV [4].

Recently, the outbreak of hepatosplenomegaly disease has been increased in chickens, although avian HEV is considered to be the major causative agent, fowl adenovirus (FAdV), reticuloendotheliosis virus (REV), avian leukemia virus (ALV), Marek’s disease virus (MDV) and chicken infectious anaemia virus (CIAV) can also cause hepatosplenomegaly and immunosuppression, which may facilitate the popularity of avian HEV [4, 5], hence, the continual epidemiological investigation of avian HEV is necessary. In the present study, the suspected cases of avian HEV infection were diagnosed, and the molecular epidemiology of avian HEV in chickens was characterized in Shandong Province between 2020 and
2021. These results will enhance the current understanding of the genetic diversity of avian HEV and provide new insights into the prevent control strategies of this disease.

Methods

Samples

In this study, 18 clinical cases (10 dead laying hens and 8 dead broiler breeders, respectively) showing severe hepatosplenomegaly, rupture and bleeding were collected, these samples were from Taian, Dezhou, Zibo, Liaocheng, Heze, Linyi, Jinan, and Jining cities in Shandong province farm the ages of onset chickens ranged from 17 to 25 weeks, and there was a significant decrease in laying, hatching and survival rate of chicks. The liver and spleen were collected and divided into two parts. One part was homogenized with sterile PBS, then subjected to freeze-thaw cycles for three times, and the supernatant was collected after 12,000 rpm centrifugation for 5 min and stored at -80°C for virus detection. The other part was fixed with 10% formalin solution, routinely processed and stained with HE method for histopathological examination.

The nucleic acid extraction and virus detection

The nucleic acid (RNA/DNA) was extracted from the treated samples using the Simply P Virus DNA/RNA Extraction Kit (Bioer, Hangzhou, China) according to the procedure. Some RNA samples were reverse transcribed into cDNA by ReverTra Ace qPCR RT Kit (TOYOBO, Shanghai, China) for the detection of avian HEV, ALV and REV by PCR method. The rest were used for the detection of MDV, FAdV and CIAV viruses. The primers of detected viruses are shown in Table 1, and the primer for avian HEV is referred to previous study [6]. According to the instructions of 2 × Accurate Taq Master Mix (Dye Plus) (Accurate Biotechnology, Hunan, China), the total volume of reaction system was 20 µL, and thermal cycling conditions were as follows: 94°C for 30 s, 35 cycles of 98°C for 10 s, 54–60°C for 30 s, and 72°C for 1 min, with a final incubation at 72°C for 2 min.
Table 1
The primers used in the study.

| Primers | Sequence (5'-3') | Product size (bp) | Annealing temperature (°C) |
|---------|-----------------|-------------------|--------------------------|
| CIAV-F  | CAGAATTCCCACCTCAAGCGACTTCGAC | 582               | 54                       |
| CIAV-R  | ATGTCGACGGGGCTGAAGGAT         |                   |                          |
| REV-F   | CATACTGAGCCAATGGTT           | 300               | 54                       |
| REV-R   | AATGTTGTAGCGGAAGTACT         |                   |                          |
| MDV-F   | TCATCAGGGTCTCCCGTCACCT       | 1005              | 55                       |
| MDV-R   | AGAGATGTCTCAGGAGCCAGAG       |                   |                          |
| FAdV-F  | AATTTCGACCCCATGACGCGCCAGG    | 508               | 56                       |
| FAdV-R  | TGGCGAAAGGCGTACGGAAGTAAGC    |                   |                          |
| ALV-J-F | GGATGAGGTGACTAAGA            | 512               | 56                       |
| ALV-J-R | CGAACAAAGTAACACACG           |                   |                          |
| HEV-F1  | TCGCCT(C)GGTAAT(C)ACA(T)AATGC | 278               | 60                       |
| HEV-R1  | GCGTTTC(G)CCG(C)ACAGGT(C)CGGCC |             |                          |
| HEV-F2  | ACA(T)AATGCT(C)AGGGTCACCCG   | 242               | 56                       |
| HEV-R2  | ATGTACTGA(G)CCA(G)CTG(C)GCCGC |               |                          |

Phylogenetic analysis of avian HEV isolates

The positive products were cloned to pMD18-T vector (Takara, Dalian, China) and were sent to Sangon Biotech (Shanghai, China) for sequencing. Sequences of avian HEV isolates generated in this study were submitted to the GenBank under accession numbers MZ231098 to MZ231108. The phylogenetic and molecular evolution of these HEV isolates were analyzed using MEGA version 6 [7] by Maximum Likelihood tree method, and the comparison of sequence identity was performed using MegAlign software (DNAStar, Madison, United States).

Results

Gross and microscopic lesions

The major gross lesions of HEV suspected cases were concentrated in the liver and spleen. In the collected samples, there were significant enlargement, rupture, and bleeding spots of liver (Fig. 1A), spleen was enlarged with spots of bleeding and necrosis foci on the surface (Fig. 1B). Histopathological lesions showed that hepatocellular necrosis and hemorrhagic foci in liver tissue, with massive heterophils
and lymphocytes infiltration around portal areas (Fig. 2A), necrosis of liver cells and amyloid deposition with a small amount of red blood cells and macrophage infiltration (Fig. 2B), the reduction the of number of lymphocyte in the spleen and extensive amyloid deposition (Fig. 2C).

**Virus detection**

It was found that 11 out of 18 samples had HEV infection after PCR detection, with a positive rate of 61.11%. It was interesting that all positive samples were mixed infections, including 2 cases of HEV and CIAV co-infection, 1 case of HEV and FAdV co-infection, 5 cases of triple infection, and 3 cases of quadruple infection (Table 2). In addition, virus infections were also observed in another 5 samples, they were mainly the mixed infections of CIAV, FAdV, and MDV.

| The types of mixed infection | The number of mixed infection cases | Viruses          |
|----------------------------|-----------------------------------|------------------|
| Co-infection              | 2                                 | HEV, CIAV        |
|                           | 1                                 | HEV, FAdV        |
| Triple infection          | 4                                 | HEV, CIAV, FAdV  |
|                           | 1                                 | HEV, CIAV, MDV   |
| Quadruple infection       | 3                                 | HEV, CIAV, FAdV, MDV |

**Sequence homology analysis and phylogenetic tree construction**

The ORF2 genes of 11 HEV isolates were amplified for sequencing and alignment. The result showed that the nucleotide homology between 11 samples was 97.6% – 100%, and the highest nucleotide homology was observed between 11 HEV isolates and others belonged to Orthohepevirus B (75.9% – 83.7%). However, the homologies with Orthohepevirus A, Orthohepevirus C, and Orthohepevirus D were low, ranging from 48.2–56.7%. Hence, we further analyzed the nucleotide homology between 11 HEV isolates and the different genotypes viruses belonged to Orthohepatic B. It was found that these isolates in this study had low homology with the 4 reported genotypes, ranging from 75.9–83.7%, among which the homology with genotype 1 was the highest (79.2% – 83.7%) (Table 3), indicating that the 11 avian HEV isolates in this study may be a novel genotype virus.
Table 3

| Isolates | Different genotypes of avian HEV* (% identity) |
|----------|-----------------------------------------------|
|          | Genotype 1       | Genotype 2       | Genotype 3       | Genotype 4       |
| 1 to 11  | 79.2–83.7        | 75.9–80.0        | 78.4–83.3        | 78.8–80.0        |

* The reference viruses of different genotypes were showed in the supplementary table 1.

The 11 HEV isolates obtained in this study have been submitted to NCBI (accession No. MZ231098 to MZ231108), and the phylogenetic tree was constructed with other known HEV virus strains of different genotypes. The result demonstrated that the 11 isolates did not share a branch with any known genotypes, and they constituted a single branch (Figure 3). The genetic evolution analysis further suggested that these isolates in the study were a novel genotype.

Discussion

HEV was first reported in Western Canada in 1991 and subsequently caused outbreak in the United States, Australia and the United Kingdom [8, 9]. It has been reported that severe HEV infections have occurred in several provinces in China since 2016 [10, 3, 11]. In recent years, outbreaks of BLS and HS syndrome in chicken flocks have gradually increased in Shandong province [10, 12]. In order to investigate the causes of BLS and HS syndrome, and the epidemic characteristics of avian HEV in Shandong province, 18 cases of hepatomegaly and splenomegaly were collected for histopathology examination and pathogen detection, including HEV, MDV, FAdV, ALV, CIAV, and REV. Histopathological examination found that hemorrhage and necrosis in the liver, the heterophagic granulocytes and lymphocytes were infiltrated in the portal areas of the liver, which were similar to the results of lymphocytic phlebitis and periphlebitis of the liver in the artificial challenge chickens infected with avian HEV [13, 1], moreover, the reduction of lymphocytes and amyloidosis were observed in the spleen. However, due to the lack of available cell for avian HEV isolation at present, the bile samples of HEV positive chickens instead of purified virus for animal challenge experiments [13, 1], therefore, the isolation of avian HEV strain and animal experiment to obtain the accuracy of the results will be the focus of our further research. Notably, there were a large number of heterophagic granulocytes infiltration in the liver in this study, this cell was related to bacterial infection, indicating that clinical avian HEV infections may have co-infection or secondary infections. The roles of the mixed infections of multiple viruses in BLS and HS syndrome cannot be ruled out.

Actually, it has been reported mixed infections of avian HEV and ALV [4], HEV and MDV since 2016 [14], and the mixed infection rate of avian HEV and several immunosuppressive viruses was up to 58% in chickens, China, including CIAV, ALV, and REV [5], which indicated that the mixed infections of HEV and other viruses were common in chicken flocks. In the current study, further PCR detection showed that all 11 HEV positive samples were mixed infections, of which the infection rates of HEV and CIAV, HEV and
FAdV were highest. CIAV is an important immunosuppressive virus in chicken, and according to the epidemiology investigation of co-infection of vertically transmitted or immunosuppressive viruses in chickens by Li et al, CIAV had the highest detection ratio [15]. FAdV has been prevalent in poultry in recent years and caused huge economic losses to poultry industry in China [16, 17]. It has been reported that chickens infected with FAdV were more likely to be infected with immunosuppressive diseases [18], and previous study has also reported that the co-infection rate of HEV and FAdV was about 10% [5]. The problem of vaccine contaminated with CIAV and FAdV may facilitate to the serious mixed infection status [19]. However, what role have CIAV and FAdV played in the pathogenesis of avian HEV and whether they can promote the onset of HEV, these questions are worthy of further study. On the whole, this study further confirmed the pervasiveness and severity of avian HEV and other viruses in chicken flock, and the continuous epidemiological surveillance is required.

Different genotypes of avian HEV have been reported worldwide [10, 20, 21, 22]. Zhao et al. reported the complete genome sequence of the first avian HEV in 2010[3], since then, the avian HEV isolates of different genotypes have been widely reported in China, including Shandong, Jiangxi and Guangdong provinces [10, 13, 23]. In order to further determine the molecular characteristics of the 11 HEV isolates obtained in this study, nucleotide homology analysis was performed, it was found that the 11 HEV isolates belonged to Orthohepevirus B (75.9–83.7%), and genetic evolution analysis showed that all isolates located in a single branch, which was different from the known 4 genotypes strains, indicating they were a novel genotype. Through these results, we speculated that avian HEV may have been widespread in China, and the genetic background of HEV has become more and more complicated.

**Conclusions**

In conclusion, this study found a new genotype of avian HEV in Shandong province, enriched the molecular epidemiological data of avian HEV, and there were significant serious mixed infections of avian HEV and other viruses in clinical.

**Abbreviations**

PCR: Polymerase chain reaction; PBS: Phosphate-buffered saline; HEV: Avian hepatitis E virus; BLS: Big liver and spleen disease; HS: Hepatitis-splenomegaly syndrome; FAdV: Fowl adenovirus; REV: Reticuloendotheliosis virus; ALV: Avian leukosis virus; MDV: Marek's disease virus; CIAV: Chicken infectious anaemia virus.

**Declarations**

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Availability of data and materials

The viral sequences obtained in this study are deposited in GenBank under the accession numbers: MZ231098 to MZ231108. The data involving in the manuscript can be obtained from the corresponding author upon reasonable request

Authors’ contributions

KL and YZ performed the experiment and wrote the manuscript, JZ, NG, FM, SW, JL and ZZ collected and analyzed data, SL and NL conceived the study and reviewed the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of Shandong Agricultural University. There were no vulnerable populations involved, and no endangered species were used in the experiments. Farm managers gave permission for their animal samples to be used in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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**Figures**

**Figure 1**

Gross lesions of HEV-infected chickens. (A) Hemorrhage and swelling of the liver, (B) Splenomegaly.
Figure 2

Histopathological lesions of HEV-infected chickens. (A) Extensive hemorrhagic foci in liver tissue, massive infiltration of portal areas with lymphocytes and heterophils. Amplification 200×, (B) Degeneration and necrosis of liver cells, massive amyloid deposition, and infiltration of red blood cells and macrophage. Amplification 400×, (C) The lymphocyte decreased and extensive amyloid deposition in the spleen. Amplification 40×.

Figure 3

The phylogenetic tree was constructed based on the partial ORF2 gene of avian HEV and 30 reference isolates deposited in NCBI. The detailed information of reference strains were shown in the
supplementary table 1.

Supplementary Files

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