


Isolation, identification and genome analysis of an avian hepatitis E virus from white-feathered broilers in China

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ABSTRACT Avian hepatitis E virus (HEV) is the major causative pathogen of the big liver and spleen disease, hepatitis-splenomegaly syndrome, and hepatic rupture hemorrhage syndrome. Until now, there are 6 different avian HEV genotypes that infect chickens have been reported worldwide. Epidemiologic investigations of the avian HEV demonstrated that avian HEV has been widely spread in China in recent years. In this study, an avian HEV named YT-aHEV was obtained from white-feathered broilers using LMH cells by virus isolation assay in Shandong province, China. The complete genome consists of 6656-nt excluding the poly(A) tail. The isolate was highly similar to the CaHEV strain and segregated into the same branch belonging to avian

HEV genotype 3. Indirect immunofluorescence using capsid protein-specific polyclonal antibodies confirmed that YT-aHEV could establish productive infection and replicate stably in LMH cells. Furthermore, an in vivo avian HEV infection model was established successfully in specific pathogen-free chicken embryos by intravenous experiments. In the present study, we demonstrate an avian HEV infection associated with liver lesions of hemorrhage and swelling by LMH cells for the first time in a white-feather broiler flock in China. This research also provides a new diagnosis method for detection of avian HEV, which laid a foundation for the understanding of pathogenicity and molecular biology of this virus for further study.

Key words: avian hepatitis E virus, genome analysis, virus isolation, white-feathered broilers

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INTRODUCTION

Hepatitis E virus (HEV) is one of the 5 known hepatitis viruses that divides into 2 genera, *Orthohepevirus* and *Piscihepevirus*. The most studied mammalian and avian HEV belong to the genus *Orthohepevirus* which contains A, B, C, and D species (Smith et al., 2014). Avian HEV is the major causative agent of the big liver and spleen disease (BLS) (Haqshenas et al., 2001), hepatitis-splenomegaly syndrome (HSS) and hepatic rupture hemorrhage syndrome (HRHS) in chickens (Su et al., 2018b; Ritchie and Riddell, 1991). Avian HEV is a single-stranded positive-sense RNA virus without an envelope, with a genome of approximately 6.6 kb in length, consisting of 3 open reading frames (ORFs) and non-coding regions (NCRs) at the 5' and 3' ends. ORF1 encodes a

nonstructural polyprotein consisting of viral methyltransferase, papain-like protease, viral helicase and RNA-dependent RNA polymerase (RdRp) (Koonin et al., 1992). These products play important roles in virus assembly and release of viral particles. 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. ORF3 encodes a cytoskeleton-associated phosphoprotein (Zafrullah et al., 1997).

In previous studies, avian HEV has been classified into 6 major different genotypes based on full or nearly complete genomes. All of these avian HEVs originated from countries including Australia (Payne et al., 1999), Korea (Kwon et al., 2012), US, Central Europe (Haqshenas et al., 2001; Matczuk et al., 2019), China (Zhao et al., 2010), and Hungary (Morrow et al., 2008; Banyai et al., 2012). In China, the existence of avian HEV infection was confirmed by serological investigations and the first complete avian HEV (CaHEV) genome sequence was reported from broiler chickens in 2010 (Zhao et al., 2010). Furthermore, many novel

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genotypes of avian HEV have been found in different regions of China which suggesting the high diversity of the avian HEV (Su et al., 2018a; Zhang et al., 2019; Liu et al., 2020). To summarize the current epidemiological studies, it is worthy of our attention that the increase of avian HEV genotypes is accompanied by wider host susceptibility (Sun et al., 2019). Both broilers and laying hens are susceptible to avian HEV, and the feces of infected chickens is the main source of infection, while later healthy chickens are infected through the fecal-oral route. In addition, contaminated feed, drinking water, and bedding (Meng, 2013) may lead to the spread of avian HEV among chicken flocks.

The isolation of avian HEV is difficult because that there is no suitable cell line for avian HEV isolation. The existence of avian HEV infection in the relevant flocks had only been diagnosed using molecular biological methods mainly including nested reverse transcription PCR (**RT-nPCR**) and real-time reverse transcription polymerase chain reaction (**RT-qPCR**). In this study, RNA was extracted from the original liver samples for RT-nPCR and sequencing and, in parallel viral isolation by LMH cells was done. Then, avian HEV antigen in LMH cells was detected by indirect immunofluorescence assay (**IFA**). Besides, viral replication kinetics of avian HEV in LMH cells was measured by RT-qPCR. Finally, we conducted infection experiments of the SPF chicken embryos with YT-aHEV. The viruses were inoculated by 3 different routes: yolk sac, urinary bladder, and intravenous. Then, the infectivity of YT-aHEV to SPF chicken embryo was verified by testing the virus shedding of fecal samples.

MATERIALS AND METHODS

Clinical Samples

The clinical cases with liver hemorrhage and swelling occurred in a white-feathered broilers farm in Shandong Province in 2020, and the peak mortality occurred at about 20 wk of age. Given avian HEV is the causative agent of BLS disease or HSS and HRHS, we suspected the infection of avian HEV in the above flock according to the clinical symptoms. The sick and dead chickens were dissected and liver samples were collected.

RNA, DNA Extraction, and RT-nPCR

Briefly, total RNA was extracted from liver samples using a commercial kit (OMEGA, Bio-Tek, Norcross, GA) and then cDNA was reverse transcribed with random primer using an AMV RT kit (Takara, Japan) following the manufacturer's instructions. DNA was also extracted using a commercial kit (TianGen, China). Avian HEV was detected using RT-nPCR method with primers listed in Table 1. In addition, the nucleic acid was strictly tested for the presence of chicken pathogens including avian leukosis virus (**ALV**), reticuloendotheliosis virus (**REV**), chicken infectious anemia virus (**CIAV**), and fowl adenovirus (**FAdV**) according to

Table 1. Primers used in the study.

Pairs	Primer	Sequences of the primers (5'→3')
1	H-Z-F1	ATGGGAAYCAGCATGTBGATG
	H-Z-R1	TADATCCACTCCTGRTGGGT
2	H-Z-F2	ATGCAGTATGTGACYAABGC
	H-Z-R2	GCTGGYTTDCCAAATGCDGG
3	5GSP1	GCACACGCCACATCCGCAGGACGCAT
	5GSP2	GCAGGACGCATGTCGTGCAGACTATA
4	3GSP1	ATCACGTGGTGTGCGTGCCCTGCTAG
	3GSP2	TGCGTGCCCTGCTAGTTCAGTCG
5	F1-1	CTAGAAGCGGCTGCTCTAGCTGC
	R1-1	AATGATGGTGGTGCAGAAGG
6	F1-2	TCTGTGGAGCTGTTCCGTGGT
	R1-2	CACATTTACGCGCTTGTGCATAA
7	F2-1	CACTGTGGGTAACATTGTGGC
	R2-1	GTTCCGACTGCTTAGCCACCTG
8	F2-2	AGGCGGAACACGCAGCA
	R2-2	TCGTCCACAATGACCCTGC
9	F3-1	GGCTGTGTGGCATGTTCCA
	R3-1	GGTAAAGAGCCACCATCCAAT
10	F3-2	CCGTGATGGTGACTGTTGTTGTTG
	R3-2	GGCACATCTCCGCATACTC
11	F4-1	CCCTCAACATTGGAGTATGC
	R4-1	ATCTGGTACCGTGCGAGT
12	F4-2	ACATTGGAGTATGCCGAGATG
	R4-2	TTGAGCGCTCCACTGGGCT
13	F5-1	CTTGTGGCAGGTTATTATGGTG
	R5-1	AGGATTTTCATAAATAATCGCATAA
14	F5-2	ATGTCGCTGCGTGGATTGCTG
	R5-2	TTAGGGTGGTGGGGGAATGTC

Pairs 1 to 2 were used for avian HEV identification.

Pairs 3 to 4 were used for RACE.

Pairs 5 to 14 were used to amplify five fragments of the near complete avian HEV genome according to reference (Zhao et al., 2010).

the published methods (Luan et al., 2016; Li et al., 2017; Dong et al., 2019).

Virus Isolation

The LMH cells were cultured in Dulbecco's modified Eagle's medium (**DMEM**; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (**FBS**; Gibco, Carlsbad, CA) at 37°C in a 5% CO₂ incubator. The original liver samples were homogenized in 1mL of PBS (0.01M, pH 7.2–7.4) and centrifuged at 10,000 *g* for 2 min, then collected the grinding fluid of the liver samples and filtrated through 0.22- μ m filter. Then, the sterile filtrates were inoculated into LMH cell monolayers grown in 12-well plates following adsorb period for 2 h. Uninfected LMH cells were used as a negative control. After adsorption for 2 h at 37°C, cells were washed 3 times with PBS (0.01M, pH 7.2–7.4) to remove nonadsorbed virus and culture medium was replaced with low-serum medium (1% FBS/DMEM). After 3 blind passages of infected cells, the culture supernatants were harvested and stored at –80°C for further analysis.

Indirect Immunofluorescence Assay

IFA was performed to further verify the isolated virus followed a standard procedure. In detail, the cell supernatant in the culture plate was discarded, and immediately fixed with paraformaldehyde at 4°C overnight. After that, permeabilized with 0.25% Triton X-100 for 15 min at room temperature. Then, cells were washed

3 times with PBS (0.01M, pH 7.2–7.4) and incubated with primary antibody (1/800 rabbit anti-capsid protein-specific polyclonal antibody) for 1 h at 37°C. After incubation, the cells were labeled with a TRITC-conjugated goat anti-rabbit IgG secondary antibody H&L (Abcam, UK) for 1 h at 37°C. Finally, the cells were washed 3 times again with PBS (0.01M, pH 7.2–7.4) and observed under fluorescence microscope.

The avian HEV capsid protein-specific polyclonal antibody was prepared in our laboratory. Briefly, ORF2 gene of an avian HEV strain (VaHEV, GenBank No. MG976720) was amplified and inserted into pET32-a (+) prokaryotic expression vector, and transformed into BL21 *Escherichia coli* (*E. coli*) cells to obtain the recombinant protein named ORF2-(His)₆. Rabbits were injected with the purified recombinant protein ORF2-(His)₆ mixed with Freund's complete adjuvant to prepare the anti-ORF2 polyclonal antibody. Western-blotting (WB) was performed to evaluate the specificity and sensitivity of prepared antibody with the established protocol.

Concentration, Purification, and Electron Microscopic Observation of Virus

LMH cell culture was centrifuge at 8000 × *g* to remove the cell fragments. Transfer the supernatant to a new bottle and slowly add PEG6000 to make a final concentration of 8% (M/V). Then, the mixture above centrifuge for 60 min at 10000 × *g* and suspend the pellets in 20 mL of phosphate buffered saline (PBS, 0.01M, pH 7.2–7.4). After that, separate viral particles of avian HEV by sucrose density gradient centrifugation.

The virus particles were observed by transmission electron microscopy using the copper mesh suspension method. The virus suspension was placed on the copper mesh with 10 μL drops and left for 10 min at room temperature in a dry environment. Ten μL of 2% phosphotungstic acid solution (pH = 6.8) was placed on the copper mesh and the mesh was negatively stained at room temperature for 5 min, dried at room temperature, observed under transmission electron microscopy and photographed.

Genomic Amplification of YT-aHEV

Viral RNA was extracted from infected LMH cellular supernatants using a commercial kit (OMEGA, Bio-Tek, Norcross, GA). The primer sequences were designed according to reference and sequences of avian HEV strains published on GenBank. All primers were listed in Table 2. The rapid amplification of cDNA ends (RACE) technique was employed to identify both the extreme 3' and the extreme 5' genomic sequence. For 3' RACE, the RNA template was firstly reverse-transcribed by 3'CDS Primer (Vazyme, China). Then, 10 × Universal Primer Mix (Vazyme) and avian HEV gene specific forward primer 3GSP1 were used in the first-round of PCR amplification. Afterward, nested Primer (Vazyme) and avian HEV gene specific inner forward primer 3GSP2 were employed in the second-round of nested PCR. For 5' RACE, the RNA template was firstly reverse-transcribed by 5'CDS Primer (Vazyme). The first-round PCR was done with primer 10 × Universal Primer Mix (Vazyme) and gene specific forward primer 5GSP1 (Vazyme). After then, Nested Primer (Vazyme) and avian HEV gene specific inner forward primer 5GSP2 were employed in the second-round of nested PCR. All PCR products were purified using the Gel Band Purification Kit (OMEGA, Bio-Tek, Norcross, GA) and cloned into pMD18-T vector (TAKARA) or pEASY-Blunt vector (TransGen, China) followed by sequencing (Tsingke, China).

Sequencing and Phylogenetic Analysis

Multiple sequence alignments were carried out with the Lasergene-MegAlign software package (DNASTAR, Inc., Madison, WI). A phylogenetic analysis was constructed using MEGA 6.0 software with the Maximum-Likelihood (ML) method with 1,000 bootstrap replicates. The sequences obtained in this study were deposited in GenBank with the accession numbers as MZ736614, and the avian HEV reference strains used in this study are shown in Table 2.

Table 2. Avian hepatitis strains used in this study.

Gen Bank No.	Species	Genotype	GenBank No.	Species	Genotype
KX387866	A	8	JN998606	C	2
KX387867	A	8	GU345042	C	1
KX387865	A	8	JQ001749	D	1
KT818608	A	7	MG976720	B	New-Genotype
KJ496144	A	7	MN562265	B	New-Genotype
AB291955	A	3	AM943646	B	3
FJ527832	A	3	GU954430	B	3
AB573435	A	5	MK050107	B	3
AB856243	A	6	KF511797	B	4
FJ763142	A	4	JN997392	B	4
AB480825	A	4	JN597006	B	1
AJ272108	A	4	KC454286	B	1
M74506	A	2	AM943647	B	1
D11092	A	1	EF206691	B	2
M73218	A	1	AY535004	B	2
AF076239	A	1	KM377618	B	2

Replication Kinetics of the Isolate in LMH Cells

A strain of avian HEV was isolated and its infectivity was determined using Reed-Muench method in LMH cells. To further confirm the infectivity and replication stability of YT-aHEV in LMH cells, avian HEV with 100 TCID₅₀ was inoculated into blank LMH cells. Since YT-aHEV could not induce cytopathic effect (CPE) in LMH cells, therefore, the infected LMH cells were passage through 5 generations, and LMH cellular supernatants of each generation were collected and RT-qPCR assay was performed for the analysis of virus replication dynamics in cell culture.

Infection Experiments of the SPF Chicken Embryos With YT-aHEV

To investigate the infectivity of YT-aHEV, specific pathogen-free (SPF) chicken embryo (SPAFAS poultry company; China) were used for inoculation experiment. SPF chicken embryos were inoculated with YT-aHEV obtained from LMH cells via yolk sac, urinary bladder and intravenous respectively (Table 3). SPF chicken embryos were inoculated with sterile PBS (0.01M, pH 7.2–7.4) used as a control group. Each chicken embryo inoculated with a viral dose of 50 TCID₅₀. After hatch, feces of chickens were collected immediately and monitored for virus shedding by both RT-nPCR method and viral isolation as previously described. Based on monitoring results, 5 feces samples were selected randomly from the feces which were positive for virus isolation. Viral genome of these 5 feces samples were sequenced using gene special primer pairs 5 to 14 (Table 1) and compared with the inoculated viruses.

RESULTS

Clinical Symptoms and Laboratory Diagnosis Tests

The disease occurred in a white-feathered broilers farm characterized by liver hemorrhage and swelling (Figure 1) with the death peak of sick chicken flocks appearing at 20

wk of age. To investigate the causative agent in this clinical case, RT-nPCR or qPCR was performed to detect avian HEV, ALV, FAdV, CIAV, and REV. The results showed that partial avian HEV ORF2 fragments in liver samples were amplified using RT-nPCR while ALV, FAdV, CIAV, and REV were not detected in the samples. Furthermore, the amplification products were sequenced and showed that the sequences shared up to 92% identity with known avian HEVs in the GenBank database.

Virus Isolation in LMH Cells and Indirect Immunofluorescence Analysis of Infected LMH Cells

The sterile filtrates of liver samples were inoculated into LMH cells for virus isolation and tested using RT-nPCR with avian HEV specific primers. The results showed that an avian HEV strain was successfully isolated by LMH cell culture system (Figure 2). In addition to the above RT-PCR method, IFA using anti-capsid protein-specific polyclonal antibody was performed. Specific red fluorescence was observed in positive wells, and fluorescent cells showed clear contours with a stained cytoplasm and clearly distinguished from background staining, while the negative control failed to exhibit red fluorescence (Figure 3). Those results indicated that an avian HEV strain was successfully isolated by LMH cell culture system and named as YT-aHEV.

Electron Microscopy of Viral Particles

The concentrated and purified virus particles were verified using electron microscopy. The virus particles derived from LMH cells observed under electron microscopy presented as irregular spherical (Figure 4).

Genome Sequencing and Analysis of YT-aHEV

Amplification of whole genome sequence of the avian HEV isolate was accomplished using the avian HEV-positive LMH cellular supernatant. The complete genome of YT-aHEV was consisted of 6,656 nucleotides (nt)

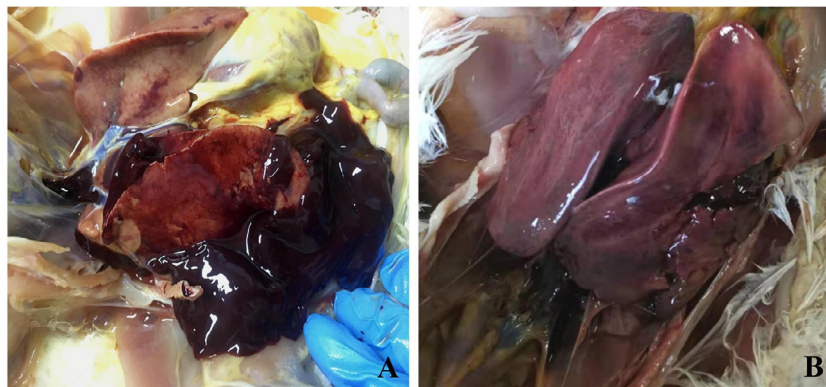


Figure 1. Clinical symptoms of broiler chicken affected with avian HEV. (A) Liver hemorrhage, (B) liver swelling. Abbreviation: HEV, Avian hepatitis E virus.



Figure 2. Electron microscopy of viral particles. The white arrows point to represent virus particles

excluding the poly(A) tail at the 3'end and contained 3 major ORFs (Figure 5). Sequence analysis revealed that YT-aHEV shared 81.4 to 97.4% nucleotide sequence identities with other avian HEV strains published in GenBank. Specifically, the ORF1 of YT-aHEV shares 80.9 to 97.3% nucleotide sequence identities and 86.3 to 98.5% amino acid sequence identities with other avian HEV strains; the ORF2 of YT-aHEV shares 81.7 to 97.4% nucleotide sequence identities and 90.9 to 99.0% amino acid sequence identities with other avian HEV strains; the ORF3 of YT-aHEV shares 85.1 to 98.5% nucleotide sequence identities and 72.4 to 96.6% amino acid sequence identities with other avian HEV strains. The homology comparison of YT-aHEV based on complete genome and each ORFs with reference strains were all listed in detail in Table 4.

Phylogenetic Analyses of YT-aHEV Complete Genome and Three ORFs

Phylogenetic analyses were performed based on the complete genome and ORF1~3 of YT-aHEV respectively. All the phylogenetic trees were estimated using

the ML method with 1,000 bootstrap replicates. Phylogenetic analysis based on the complete genome showed that YT-aHEV was located in the genotype-3 subclade of the *Orthohepevirus* B species accompanied with other reference strains isolated from chickens. Furthermore, YT-aHEV was clustered together with two avian HEVs (CaHEV and 05-5492) belonging to the same branch (Figure 6). Phylogenetic relationship based 3 major ORFs nucleotide sequence was similar with the above result (Figure 6).

Genetic Characterization of Capsid Gene of YT-aHEV

A total of 82 different sites were observed in avian HEVs, of which 5 sites were only found in YT-aHEV,

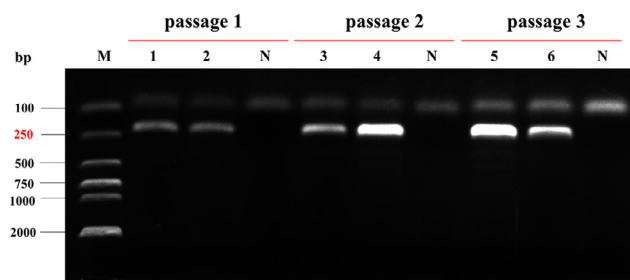


Figure 3. PCR detection of LMH cells infected with liver suspension from sick chickens. Numbers represent cells and supernatant samples of different generations. Numbers 1,3,5 representing the supernatant samples; the numbers 2,4,6 represent the cell samples; N represent negative control.

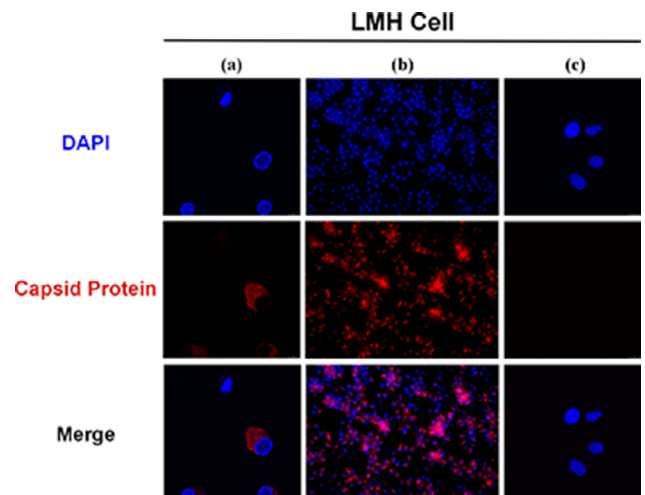


Figure 4. Immunofluorescence assay of LMH cells infected with liver suspension from sick chickens. (A), (B) LMH cells infected with liver suspension with anti-capsid protein-specific polyclonal antibody, (C) uninfected LMH cells as negative control.

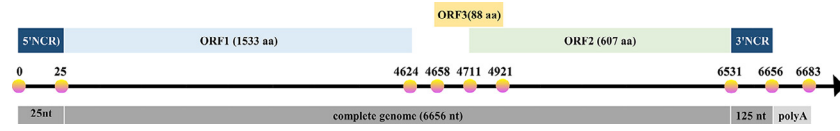


Figure 5. The complete genome of the new isolate.

Table 3. Animal experiment design.

Group	Method	Time of inoculation (age of embryos)	Dose (TCID ₅₀)
A	yolk sac	7	50
NC-A	yolk sac	7	PBS
B	urinary bladder	7	50
NC-B	urinary bladder	7	PBS
C	intravenous	14	50
NC-C	intravenous	14	PBS

namely 150S, 162F, 163Y, 165H, 238C (Figure 7). Further comparison revealed that 3 sites, 163T, 165F, and 238D, were distinct from both other known avian HEVs and mammalian HEVs (Figure 7). According to previous studies about the antigenic domain in the capsid gene (Haqshenas et al., 2001; Guo et al., 2006), compared with other avian HEVs, the new isolates had no mutation sites in antigenic domains I~IV. Only one

mutation site Q473M existed in YT-aHEV that is consistent with CaHEV and 05-5492 and different from other avian HEVs (Figure 8). On the other hand, in the three N-linked glycosylation sites, ²⁵⁵NLS appeared in all avian HEVs including YT-aHEV; the ⁵¹⁰NST appeared in the YT-aHEV and is consistent with Korea (JN597006), JY-F2 (KC454286), prototype aHEV (AY535004), CaHEV (GU954430) and HU16773 (JN997392), China (MN562265), China (MK050107); ⁵²²NGS appeared in YT-aHEV and most references strains except VaHEV(MG976720).

Determination of Viral Replicate Kinetics in LMH Cells

LMH cells were inoculated with quantified virus and passaged through another 5 generations. The replicate

Table 4. Homology comparison of complete genome of YT-aHEV with known avian HEV strains.

Orthohepevirus B HEV strains GenBank No./Country	ORF1, %		ORF2, %		ORF3, %		Complete genome Nucleotides
	Nucleotides	Amino acids	Nucleotides	Amino acids	Nucleotides	Amino acids	
05-5492/AM943646/Hungary	97.1	98.5	97	98.7	98.5	96.6	97.1
06-561/AM943647/Australia	81.8	93.2	83.7	97.9	92.8	90.9	82.4
AY535004/USA	80.9	93.4	84	98	92.8	87.5	82
CaHEV/GU954430/China	97.3	98.4	97.4	99	98.1	96.6	97.4
EF206691/USA	81.7	93.2	83.4	97.4	92.8	87.5	82.4
GI-B/KM377618/Korea	81.4	93.3	83.6	97.4	92	86.4	82.2
HEV/MN562265/ Gansu, China	81.1	93	82.3	97	86.3	75.9	81.4
HU-16773/JN997392/Hungary	83.9	95.6	86.1	98.7	95.5	92	84.6
HH-F9/JN597006/Korea	81.8	93	84.9	97.7	92.4	89.8	82.8
JY-F2/KC454286/Korea	81.1	92.6	83.9	97.5	92.4	90.9	81.9
KF511797/ Taiwan, China	83.3	95.2	84.6	98.2	94.7	93.2	83.9
CaHEV/MK050107/GDSZ0-Guangdong, China	85.3	94.9	85.8	98.4	95.8	93.2	85.5
VaHEV/MG976720/Hebei, China	82.3	86.3	81.7	90.9	85.1	72.4	82.2

Abbreviation: Hepatitis E virus.

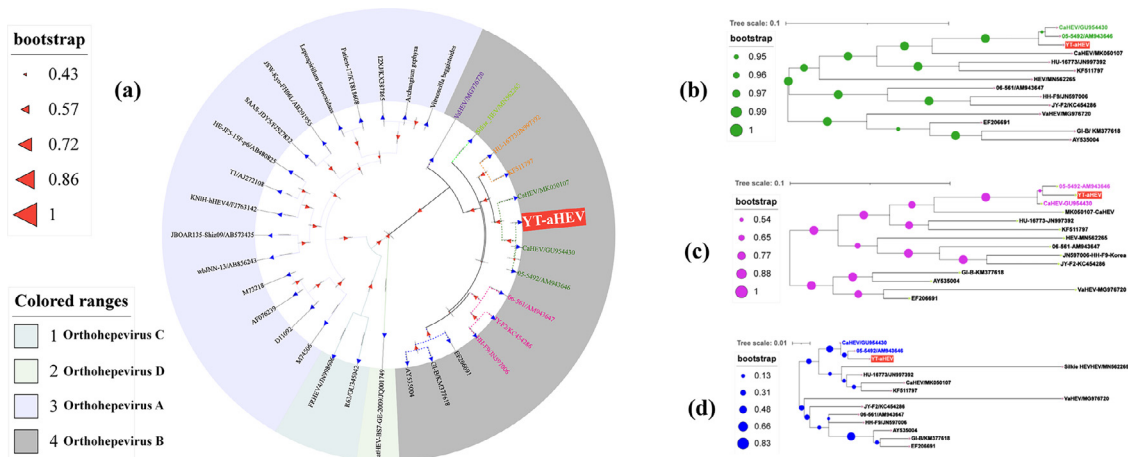


Figure 6. Phylogenetic analysis of YT-aHEV. (A) Phylogenetic tree based on the complete genomic nucleotide sequences (include play A tail) of YT-aHEV and reference avian HEVs; (B) phylogenetic tree constructed based on ORF1 nucleotide sequence; (C) phylogenetic tree constructed based on ORF2 nucleotide sequence; (D) Phylogenetic tree constructed based on ORF3 nucleotide sequence. The tree was constructed by the ML method with 1,000 bootstrap replicates using MEGA 6.0. The avian HEV isolated in the study are shown with red background.

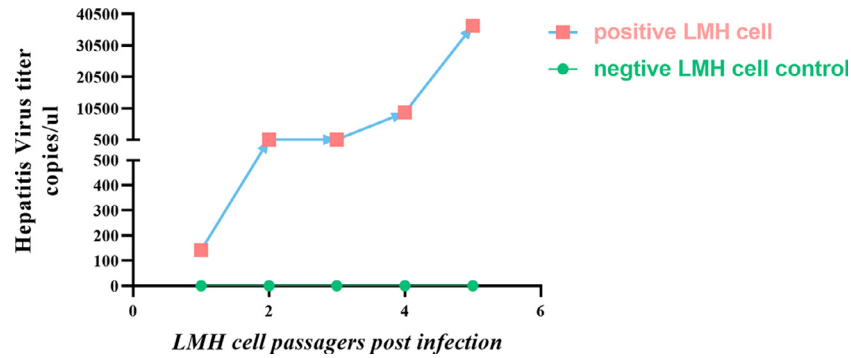


Figure 9. Replication kinetics of the new isolate in LMH cells.

from those feces samples, indicating a successful infection of avian HEV through intravenous of SPF chicken embryos. Nucleotide homology between viruses genome obtained from five avian HEV-positive fecal samples and the original inoculated virus was 99.3 to 99.5%. The nucleotide homology between those five viruses genome derived fecal samples was also 99.3 to 99.5%.

DISCUSSION

Avian HEV was firstly reported in United States and Canada in 1991 (Ritchie and Riddell, 1991), and spread around the world including Australia, Korea, US, and other countries. The first complete genome of avian HEV was obtained from broiler breeder with the clinical signs and pathological changes in 2010, China (Zhao et al., 2010). However, due to the lack of effective in vitro culture systems, the isolation and quantification study of avian HEV was very difficult which also limited the pathogenicity and in-depth study of avian HEV.

In general, pathogenic avian HEV mainly appeared in broiler breeder hens and laying hens of 30 to 72 wk age and caused mortality around 1% (Ritchie and Riddell, 1991; Billam et al., 2005). Recently, there were obvious clinical symptoms were discovered in a white-feather broiler breeder from chicken flocks with liver hemorrhage and swelling in China, and the peak incidence was at 20 wk of age. To investigate the pathogens associated with observed symptoms in this clinical case, relevant viruses were strictly detected strictly in those collected samples. Partial ORF2 gene sequences were amplified from total nucleic acids using nPCR approach, the PCR result was further confirmed by Sanger sequencing. The clinical samples were inoculated into the LMH cells for virus isolation. The viral isolation was confirmed by performing IFA. Interestingly, the successful infection of avian HEV in LMH cells was confirmed by both RT-nPCR and IFA assay. The dates of further viral infection experiment showing the isolated virus, designed as YT-aHEV, could replicate stably in LMH cells, and the copy number of virus demonstrated a statistically significant increasing trend along with cell passage, indicating a productive infection was established during this progress. To the best of our current

knowledge, this is the first report of successful isolation of an avian HEV strain in LMH cells.

To gain more information of genetic properties of the new avian HEV strain, the viral RNA was extracted from the cellular supernatant and amplified to obtain the whole genome sequence of YT-aHEV. The homology comparison revealed that YT-aHEV shared 97.4% homology with CaHEV isolated in 2010 in China, 97.1% homology with the European strain 05-5492, and only 81.4 to 85.5% homology with other known avian HEV strains. Genetic evolutionary analysis showed that YT-aHEV was closely related to CaHEV and 05-5492 strains and recognized as genotype 3, indicating a genetic complexity for avian HEV in poultry flocks in China. A total of 82 different sites of capsid gene were observed in YT-aHEV, among which 5 sites were only found in YT-aHEV. It was worth noting that three sites, 163T, 165F and 238D, were unique to avian HEVs and mammalian HEVs. Sequence alignment also revealed that the Q473M mutation occurred in YT-aHEV, CaHEV and 05-5492. The mutations found in this study may lead to increased pathogenicity and be the critical factors to change the antigenicity of virus.

In order to evaluate the infectivity of YT-aHEV in chickens, YT-aHEV proliferated in LMH cells were inoculated into chicken embryos in 3 different ways. The results indicated that about 60% chickens were infected through intravenous, while chickens were infected through yolk sac or urinary bladder with a low success rate. In addition, avian HEV can be reisolated by using LMH cells in feces, which indicated that avian HEV infection has been successfully established in these chickens. Full genome analysis of the viruses in the fecal samples indicates quite high homology of avian HEV in different chickens infected by YT-aHEV. In addition, the nucleotide homology between reisolate viruses and the inoculated viruses up to 99.3 to 99.5%. There were no insertions or deletions of nucleic acid fragments, except for mutations in individual bases. The establishment of the animal infection model will lay a foundation for the subsequent pathogenicity experiments of the strain.

In conclusion, this study demonstrated that a classical avian HEV strain named YT-aHEV belongs to genotype 3, and it was the mainly causative agent of liver hemorrhage and swelling in broiler breeder chicken flocks in

China. Most importantly, this study confirms the existence of avian HEV infection in a broiler chicken flocks by virus isolation using LMH cells for the first time. The date also showed that YT-aHEV could replicate stably in LMH cells, which provides an *in vivo* model for in-depth research and is conducive to virus diagnosis and prevention. So far, the invasion pathway and pathogenic mechanism of avian HEV are not fully understood. The pathogenicity of YT-aHEV, the process of virus invasion and host corresponding receptors will be investigated in the further research.

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Ethical statement: The animal care and use protocol were approved by the Shandong Agricultural University Animal Care and Use Committee (SDAUA-2016–002). All the experimental animals of this study were cared for and maintained throughout the experiments strictly following the ethics and biosecurity guidelines approved by the Institutional Animal Care and Use Committee of Shandong Agricultural University.

DISCLOSURES

No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that was not previously submitted and not under consideration for publication elsewhere, in whole or in part.

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