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Original article

Isolation, identification and attenuation of a pathogenic duck hepatitis virus type 1 in China, and complete genomic sequence comparison between the embryo-passaged, attenuated derivatives and their parent strain

X. Liu[1,2], X. Kong[2]

[1] College of Life Science, Huaibei Normal University, Dongshan Road 100, Huaibei City 235000, PR China
[2] Division of Avian Infectious Diseases, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, Haping Road 678, Harbin 150001, PR China

Abstract

Duck viral hepatitis (DVH) is an acute and fatal disease of young ducklings characterized by rapid transmission and damages. The most important agent of DVH is duck hepatitis virus 1 (DHV-1). The effective control of DVH was achieved by active immunization of 1-day-old ducklings with an attenuated DHV-1 virus vaccine. However, the attenuated virus might reverse to virulence. In this study, a DHV-1 strain, Du/CH/LBJ/090809, was identified and its genomic sequences were determined. The genome of Du/CH/LBJ/090809 is composed of 7,692 nt excluding poly A and the virus was clustered into genotype A by comparing with other referenced DHV-1 strains. Du/CH/LBJ/090809 could lead to 30% mortality of 10-day-old specific pathogen free (SPF) ducklings. The virus was passaged serially in SPF chicken embryonated eggs and three viruses, passage 16 (P16), P29 and P40, were selected for genomic analysis. P29 and P40 were used to evaluate the attenuation in duckling by inoculating the virus to 10-day-old SPF ducklings. Results of vaccination-challenge assay showed that the inactivated virus P40 could evoke protection against the pathogenic parent virus. Nucleotide and amino acid sequences of the genomes of Du/CH/LBJ/090809, P16, P29 and P40 were compared. Changes both in nucleotides and amino acids, which might be contributed to the decreasing in virulence by chicken embryo-passaging of DHV-1, were observed. We speculated that these changes might be important in the adaption and attenuation of the virulent virus. Additionally, strains obtained in this study will provide potential candidate in the development of vaccines against DHV-1.

Key words: duck hepatitis virus 1, pathogenicity, attenuation, mutant site

Correspondence to: X. Liu, e-mail: liuxiaoli0365@sina.com, fax: +86 561 3803237

Introduction

Duck viral hepatitis (DVH), which was first described on Long Island in 1949 (Levine and Fabricant 1950), is an acute and fatal disease of young ducklings characterized by its rapid transmission and damages in livers of ducklings (Woolcock 2003). The morbidity is around 100% and mortality is about 90% in natural infection. Since its first outbreak in Long Island, DVH was subsequently reported in many other countries including China. And DVH has becoming one of the most important diseases in duck-raising areas all over the world.

The agent of DVH is duck hepatitis virus (DHV) and it encompasses three distinct types (DHV-1, DHV-2 and DHV-3) (Haider and Calnek 1979). No antigenic relationships have been found between the three types (Woolcock 2003). Among the three types of DHVs, DHV-1 is the most widespread and virulent one and belongs to the genus *Avihepatovirus* in the family Picornaviridae (Lefkowitz et al. 2018). Thus far, DHV-1 strains and their genomic sequences have been reported world widely (Kim et al. 2007, Tseng et al. 2007, Ding and Zhang 2007, Wang et al. 2008). DHV-3 was isolated in Korea and China (Kim et al. 2007, Li et al. 2013), while the nucleotide sequences specific to DHV-2 were first reported from UK (Todd et al. 2009). The genome of DHV-1 is single-stranded, poly-adenylated, positive--sense RNA of approximately 7,700 nt with a virus-encoded protein covalently linked to the genomic 5′-end (Kim et al. 2007). It has a large open reading frame (ORF) encoding a polypeptide of 2,249 amino acids, which is flanked by 5′- and 3′- untranslated regions (UTRs). The polypeptide organization of the DHV-1 was NH_2-VP0-VP3-VP1-2A1-2A2-2B-2C-3A--3B-3C-3D-COOH.

The effective control of DVH, by a live attenuated virus vaccine, has been demonstrated by Crighton and Woolcock (1978). Vaccines against DHV-1, including egg-yolk antibody (intramuscular injection, eg. Yagan Teling, Hongkong YaXin) and live vaccine (hypodermic injection, A66, Nanjing Tianbang), are available. However, disease caused by DHV-1 remains popular in China, that might come from the reversion of virulence and antigenic alteration. Hence, understanding of the differences between the prototype DHV-1 and the attenuated viruses may be of great interest to address this problem.

There are reports of genomes of the different DHV-1 strains and attenuated viruses; however, comparison between genomic sequences of the attenuated strains and their parent virus is unavailable. In this study, we isolated a DHV-1 strain, termed as Du/CH/LBJ/090809 (JF828997), and the strain was attenuated by serial passaging in SPF embryonated chicken eggs. The genomes of pathogenic strain and three of the passages (16, 29 and 40) were determined and compared. The protective efficacy of inactivated P40 against virulent DHV-1 was also evaluated. Strains and results in this study will provide useful tools in illustrating the attenuation of DHV-1 by passaging in SPF chicken embryonated eggs and provide candidate for the development of a new vaccines.

Materials and Methods

Virus isolation and identification

Livers of the dead ducklings suspected to be infected with DHV-1 from a duckling flock in Beijing were pooled and homogenised by making 10% weight/volume tissue suspensions using phosphate-buffered saline (PBS), clarified by centrifugation at 1,500 g at 4°C for 10 min, and inoculated into the allantoic cavities of 10-day-old SPF embryonated duck eggs (HVRI, China) after being filtered through 0.22 μm membrane filters (Millipore products division, Bedford, MA). Blind passages were performed until the characteristic embryo changes, such as runting, edema, skin hemorrhage of embryos and dead embryo, appeared between 3 and 7 days after inoculation. The harvested allantoic fluid was first tested by hemagglutination assay (HA) and then for DEV, reovirus and adenovirus by polymerase chain reaction (PCR).

For DHV-1 virus testing, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed by using random primer as routine protocol. And P13 and P12 (Table 1) were used as specific primers to obtain the genomic sequences of DHV-1. PCR conditions included 5 min incubation at 95°C followed by 30 cycles at 94°C for 30 s, annealing at 50°C for 30 s, and 72°C for 1 min. The amplified fragment was purified and cloned into pMD18-T vector and sequenced. Finally, the determined virus was termed as Du/CH/LBJ/090809.

Pathogenicity

The titer of Du/CH/LBJ/090809 was determined by inoculation of the viruses at 10-fold dilutions into groups of five-10-day-old SPF duck embryonated eggs. The 50% embryo infectious dose (EID_{50}) was calculated by the method of Reed and Muench (Reed and Muench 1938). Thirty 10-day-old SPF ducklings (HVRI, China) were divided into 2 groups and housed in different isolators. Twenty ducklings in group 1 were infected by intramuscular inoculation with 10^6 EID_{50} of Du/CH/LBJ/090809. The remaining birds in group 2 were mock inoculated with PBS and served as control. All animal experiments were performed according

Table 1. Sequences of the primers used in amplification.

Primer[a]	Sequence(5′-3′)	Size(bp)[c]	Position[d]
DP1	TTTGAAAGCGGGTGCATGCATGGCCATTTTC	838	1-31
DP2	TCATAAGAACAGGAGTAAAAATCATCr[b]		813-838
DP3	GTGTGGCATCCACCAACTC	1603	730-748
DP4	TCTCCAGAAAAATAGGCAAAr		2313-2332
DP5	AATTTTGAGACAGCTAATGT	1569	2151-2170
DP6	TTTCATTGTCATAAAAAACCr		3700-3719
DP7	AGAAGTGGAATGGCAATTG	1573	3499-3517
DP8	ACTTCTTGCCTAACCTGCr		5054-5071
DP9	GGCCTTGAATGTTGGAATC	1430	4992-5010
DP10	TCCATAACATCCTGTCTGAAr		6402-6421
DP11	ATGGCTAAGAAAGCATCTGA	1430	6168-6187
DP12	GTCTTATACTAAATACTAACr		7578-7597
DP13	TAGTGTTGTGGGATACCC	~285	7420-7437

[a] The names of the primers.
[b] r indicated the reverse sequence.
[c] The size of the amplified fragment.
[d] The positions of the primers in the genome of the reference strain under the accession no. EU264072

to the guidelines of the Committee on the Ethics of Animals of Heilongjiang.

Adapting of Du/CH/LBJ/090809 in SPF chicken embryos

The Du/CH/LBJ/090809 strain was serially passaged by inoculating 9-day-old SPF chicken embryonated eggs (HVRI, China) by the allantoic cavity route. The allantoic fluid was harvested 72 h post-incubation and stored at -70°C. At every 5th passage starting with passage 10, the allantoic fluid was examined for DHV-1 by RT-PCR mentioned above. Viruses of different passages were designated as follows: passage 16, P16; passage 29, P29 and passage 40, P40. Virus titrations of P29 and P40 strains were determined and expressed as EID_{50}. The pathogenicities of P29 and P40 were examined by inoculating 10^6 EID_{50} virus to 10-day-old SPF duckling intramuscularly according to the guidelines of the Committee on the Ethics of Animals of Heilongjiang.

Protective efficacy of vaccination with inactivated P40

DHV-1 P40 was inactivated by adding formalin to final concentration of 0.16% (v/v) and incubating for 16 h at 37°C. The inactivated virus was emulsified with equal volume of complete Freund's adjuvant (Sigma, USA) and kept at 4°C until use. Forty 1-day-old SPF ducklings were divided into two groups. Birds in group 1 were treated with the emulsified inactivated P40 at 4 days old (intramuscular inoculation). Birds in group 2 served as control. After 32 days from the immunization, all birds were challenged with 10^6 EID_{50} pathogenic Du/CH/LBJ/090809 strain intramuscularly according to the guidelines of the Committee on the Ethics of Animals of Heilongjiang. All birds were observed daily and sacrificed 3-day and 7-day after challenging for pathological lesion.

Viral RNA extraction, RT-PCR amplification and sequencing

Total RNA was extracted and transcribed, and PCR was performed as above mentioned. Primers used in the present study are listed in Table 1. Sequences in 3′-end of the genome were obtained by using 3′-rapid amplification of cDNA ends (RACE) kits (TaKaRa, Dalian, China) according to the manufacturer's instructions. Fragments were purified and cloned into pMD18-T vector and sequenced. Sequences of different fragments were assembled and analyzed.

Genomic sequences analysis of the Du/CH/LBJ/090809, P16, P29 and P40

The nucleotide sequences of the Du/CH/LBJ/090809, P16, P29 and P40 were determined and assembled. The ORFs in the genomes were detected by using Gene

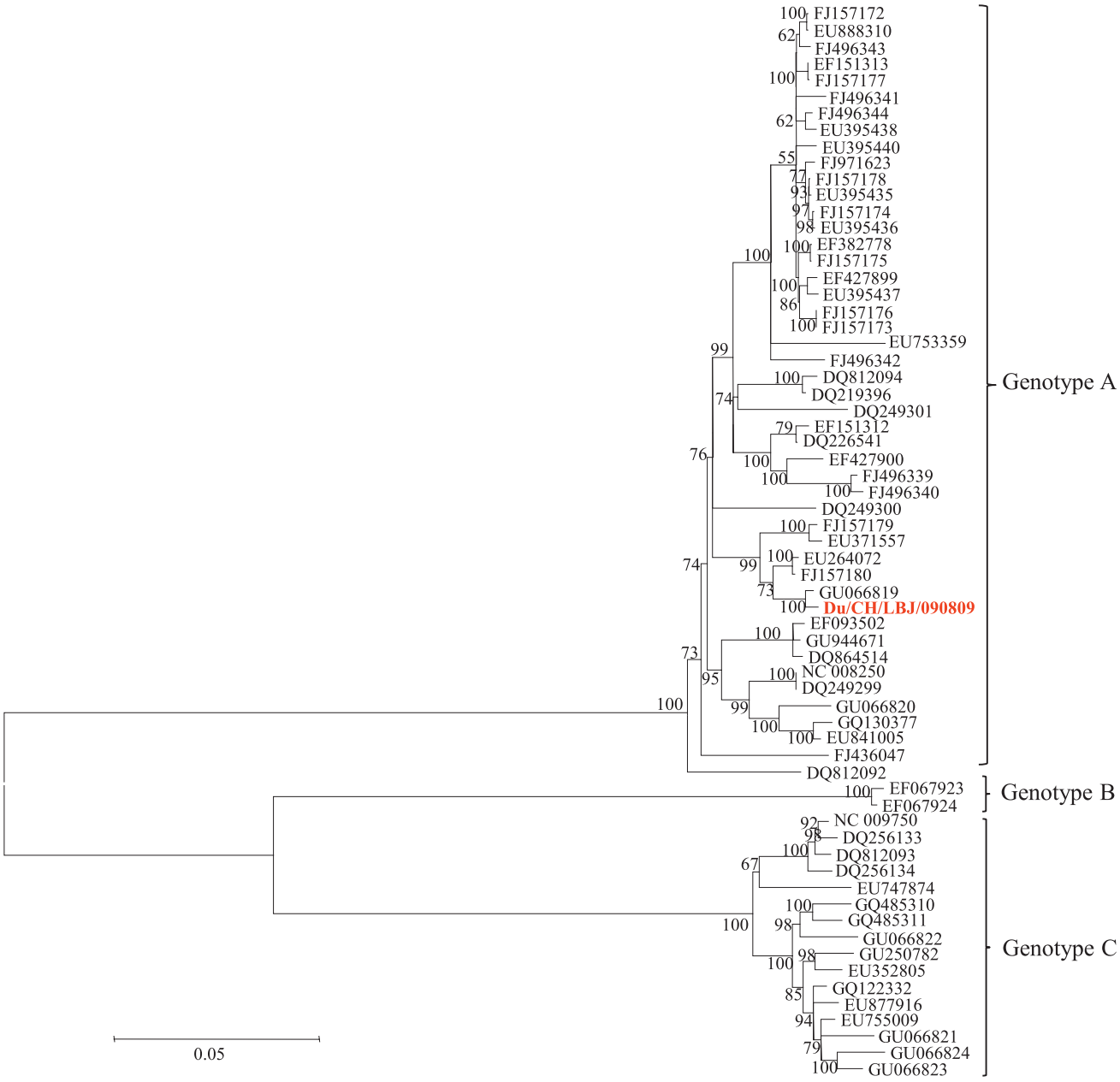

Fig. 1. Evolutionary relationship of the genomic sequences of Du/CH/LBJ/090809 with other DHV-1 strains. The phylogenetic tree was constructed using MEGA 6.0 based on the nucleotide sequences, aligning with the 64 reference strains. The accession numbers of the reference strains were indicated in the phylogenetic tree. The strain isolated in the present study was in red and bold.

Runner (version 3.00, Hastings Software, Inc., Hudson, NY, USA). The potential proteolytic cleavage sites of the DHV-1 polyprotein were predicted by amino acid alignment with the parechoviruses and other picornaviruses, and using the NetPicoRna V1.0 program (Blom et al. 1996). The phylogenetic tree was conducted by using MEGA6.0 (Kumar et al. 2016).

Accession numbers

The genomic sequences of DHV-1 Du/CH/LBJ/090809 strain and its derived strains, P16, P29 and P40, were deposited in GenBank under accession numbers of JF828997, JF828998, JF828999 and JF829000. The accession numbers of the reference strains of DHV-1 were indicated in the phylogenetic tree (Fig. 1).

Results

Virus isolation and analysis of the genome of Du/CH/LBJ/090809

A pathogenic DHV-1 strain, Du/CH/LBJ/090809, was isolated from DVH-suspected livers of ducklings.

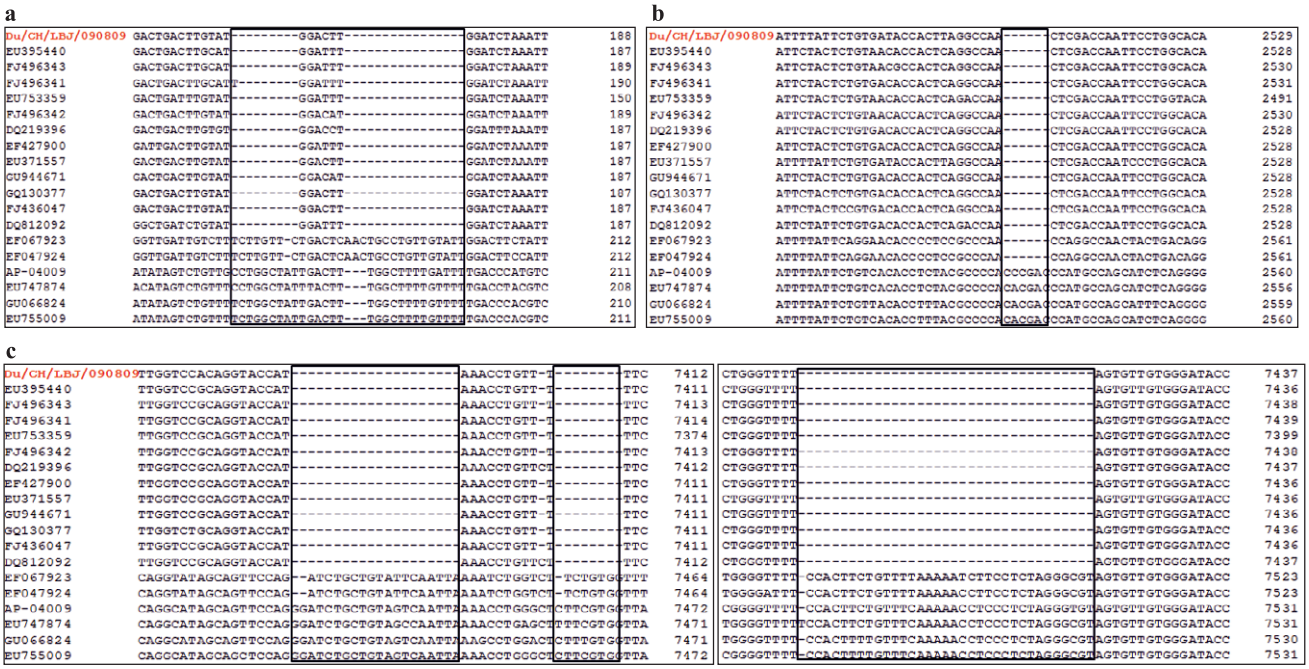

Fig. 2. Deletions in the genomic sequences observed from the alignment of Du/CH/LBJ/090809 with 18 representative DHV-1 strains. (a) The box indicated the deletion in 5`-UTR of genomes of the viruses in genotype A compared with the strains in genotype C; (b) The box indicated the deletions in VP1 gene of the viruses in genotype A compared with the strains in genotype C; (c) The boxes indicated the deletion in 3`-UTR of the viruses in genotype A compared with the those in genotype C.

The genome of the virus was determined to be 7,692 nucleotides (nt) in length, which contained a 5′-UTR of 627 nt, a single large ORF encoding a polypeptide of 2,249 amino acids and a 3′-UTR of 315 nt excluding the poly (A) tail. The polypeptide contained 12 mature products in the following order, NH_2-L-VP3-VP0-VP1--2A1-2A2-2B-2C-3A-3B-3C-3D-COOH, by comparing with other picornaviruses and being analyzed by using NetPicoRna V1.0 program.

The genome of Du/CH/LBJ/090809 was compared with referenced strains both at nucleotide and amino acid levels. Phylogenetic tree was constructed basing on the whole genomic sequences and grouped DHV-1 strains into 3 clusters (Fig. 1), and Du/CH/LBJ/090809 belonged to genotype A, the most widely prevalent genotype in China. Sequence alignment of Du/CH/LBJ/090809 and 18 selected representatives were conducted and the results indicated that the identities of strains from the same genotype were obviously higher than those of strains from different genotypes and three deletions in 5′-UTR (Fig. 2a), VP1 gene (Fig. 2b) and 3′-UTR (Fig. 2c) were detected in genome of Du/CH/LBJ/090809 compared with viruses in genotype C.

Pathogenicity of Du/CH/LBJ/090809

The EID_{50} of Du/CH/LBJ/090809 was determined to be $10^{7.16}$/0.1 ml. The morbidity and mortality of the virus to 10-day-old ducklings was determined to be 40% and 30%, respectively. The diseased ducklings infected by Du/CH/LBJ/090809 were lethargic and squat down since 24 h post-inoculation and the dead ducklings had their heads drawn back. Livers of the dead ducklings were enlarged and mottled with hemorrhages, with obvious lesions in pathological sections of livers (Fig. 3). The dead ducklings also showed swollen spleens and kidneys.

Attenuation of Du/CH/LBJ/090809 by passaging in SPF chicken embryonated eggs

The Du/CH/LBJ/090809 strain was passaged serially in SPF chicken embryonated eggs. P29 evoked characteristic changes in the inoculated chicken eggs, such as edema, and skin hemorrhage of embryos. However, P29 strain could not lead to the dead eggs. Until P40, the chicken embryonated eggs died about 70 h post--inoculation and the embryo showed severe edema, skin hemorrhage and so on. The lesions in the embryos during the passaging are shown in Fig. 4.

Pathogenicity of P29 and P40

Titers of P29 and P40 were determined to be $10^{5.9}$/0.1 ml and $10^{6.6}$/0.1 ml, respectively. No clinical signs were observed in the groups of ducklings given 10^6 EID_{50} of P29 and P40 by intramuscular amplification, which indicated that the P29 and P40 had been attenuated.

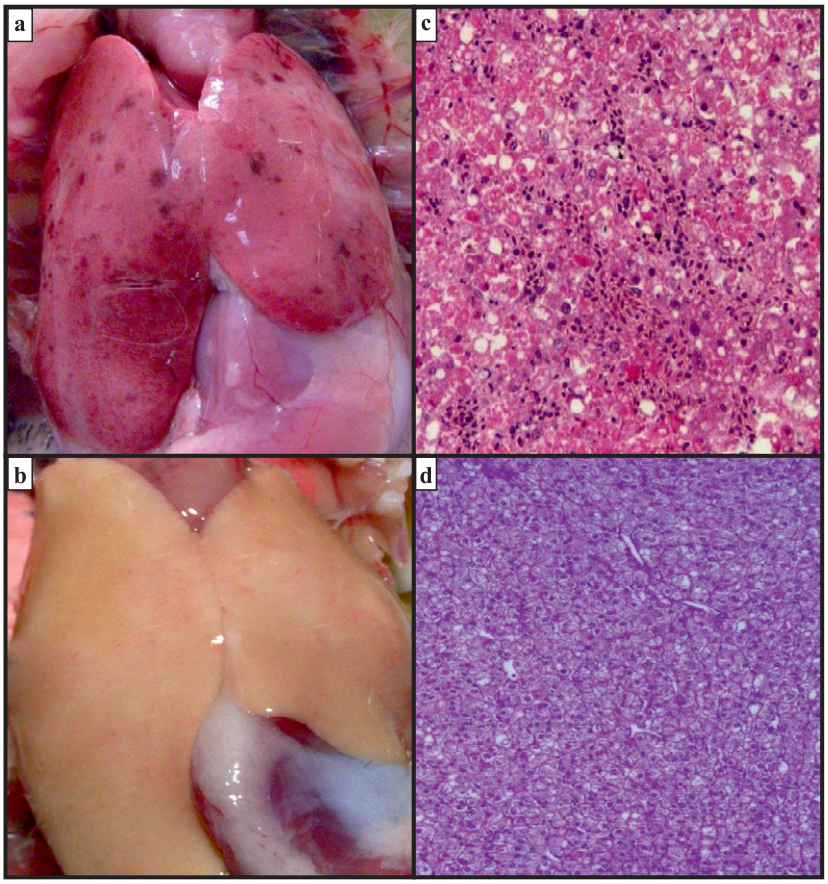

Fig. 3. The lesions and pathological sections of livers of ducklings infected by DHV-1 Du/CH/LBJ/090809 strain. (a) Liver of the dead ducklings infected with Du/CH/LBJ/090809; (b) Normal liver; (c) Histopathological changes were observed in the liver from the dead duckling that was inoculated with Du/CH/LBJ/090809 strain; (d) Histopathological examination of liver from duckling in the control group.

Protective efficacy of the inactivated P40 vaccine strain

The inactivated P40 strain was used to assess its protective efficacy against homologous pathogenic Du/CH/LBJ/090809. Neither morbidity nor mortality was observed in the vaccinated group. However, 4 birds died between 48 h and 96 h post-inoculation in control group. The results stated that the inactivated P40 strain could protect ducklings from attack of pathogenic virus Du/CH/LBJ/090809.

Genomic analysis and comparison of P16, P29 and P40

Genomic sequences of the P16, P29 and P40 were determined and compared with pathogenic Du/CH/LBJ/090809 strain. The length of the genomes of the P16, P29 and P40 were 7,692 nt excluding the poly A, the same as their parent strain. The genomic comparison indicated 10 points mutants at nucleotide level in P16 virus, leading to 5 mutants in amino acids. Thirteen points mutations were detected in P29, leading to 7 mutations in amino acids sequences. Seventeen mutations were detected at the nucleotide level in P40 virus, which led to 9 amino acids changed. However, sequence variability did not cause frame-shift. Mutations in nucleotide and amino acid levels were listed in Table 2.

Discussion

The first report of DVH appeared in 1950 and later the agent of the disease, DHV, was isolated by using embryos (Levine and Fabricant 1950). Since then, the disease was reported in many countries. Now, DHV-1 has become the most important agent to duck-raising farms and world widely distributed, especially in China (Kim et al. 2007, Li et al. 2013). In this study, a pathogenic DHV-1 strain, Du/CH/LBJ/090809, was isolated from DVH-suspected livers of ducks in China and its genome was sequenced and analyzed.

The phylogenetic analysis based on the genomic nucleotides alignment figured three genotypes of DHV-1, the same as the published results (Wang

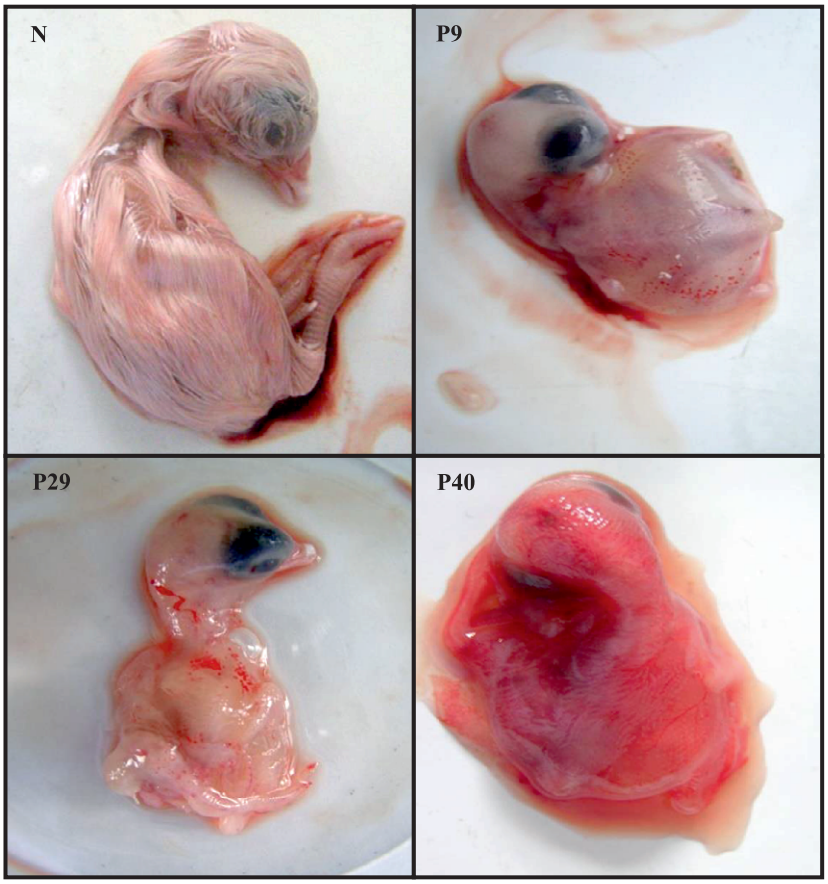

Fig. 4. Pictures of the chicken embryonated eggs during the passaging. P9, P29 and P40 indicated the chicken embryonated eggs of passages 9, 29 and 40, respectively. The normal chicken egg was indicated by 'N' as control.

et al. 2008). The isolated Du/CH/LBJ/090809 belonged to cluster A, which contained most world-widely distributed strains. We speculated that the close relationship and high identity in genomic sequences between Du/CH/LBJ/090809 and most of published reference strains in genotype A might lead to similar antigenic characterizations. Hence, it might be a potential candidate for developing new effective vaccine against DVH.

It has been reported that the DHV-1 could lead to high mortality of ducklings less than 3 weeks old. Although vaccination and antiserum against DHV-1 had been used, the DVH has become more popular, which meant new candidate strain for vaccine may be necessary. Passaging in chicken embryos is a good method for attenuation of avian viruses (Liu et al. 2007). Hence, to develop an effective vaccine strain against DHV-1, Du/CH/LBJ/090809 was attenuated by serial passage in chicken embryonated eggs. After being passaged for 40 times, one completely attenuated virus was obtained by test of attenuation. The complete protection against the attack of parent pathogenic virus of ducklings by the inactivated P40 indicated that the attenuated P40 virus would be a potential vaccine strain against DHV-1.

To understand the sequence variability of the attenuated strains and to illustrate whether the attenuated viruses had different genomes from the prototype virus, three of the middle passages during the attenuation were selected for sequencing. Mutants both at the nucleotide and amino acid levels during attenuating were detected. Only 9 mutations in amino acids were observed in P40 compared with the prototype virus and no reversion mutation from P16 to P40 was detected at amino acids level, indicating that these mutations might be useful for the adaption the virus to chicken embryonated eggs and the decrease of pathogenicity of the pathogenic Du/CH/LBJ/090809 virus to ducklings.

The polyprotein encoded by DHV-1 genome was unstable and it would be rapidly cleaved by virus-encoded proteinases to proteins involved in viral proliferation. Interestingly, none of the changed amino acids located at the cleavage sequences. That might suggest that all the functions of the proteins including proteinases would not change during passaging of DHV-1 in chicken embryos. VP1 is the most important structural protein, and the most external and dominants of all surface proteins and containing motifs that interact with cellular receptors and neutralizing monoclonal antibodies of DHV-1 (Liu et al. 2008). The inac-

Table 2. Comparison of nucleotide and amino acids equences of Du/CH/LBJ090809 and P16, P29, P40 strains.

Gene[a]	Position[b]	Du/CH/LBJ/090809	P16	P29	P40
5′-UTR	292	G	A	A	G
5′-UTR	389	T	C	T	T
L	648	C(N)[c]	C(N)	C(N)	T(N)
L	704	C(A)	C(A)	C(A)	T(V)
VP0	757	G(A)	A(T)	A(T)	A(T)
VP3	1863	G(S)	G(S)	A(S)	A(S)
VP1	2663	A(N)	G(S)	G(S)	G(S)
VP1	2733	A(K)	A(K)	G(K)	G(K)
VP1	2789	C(T)	C(T)	T(I)	T(I)
2A	2921	A(R)	G(R)	G(R)	G(R)
2C	4258	C(H)	T(Y)	T(Y)	T(Y)
2C	4549	T(F)	T(F)	T(F)	C(L)
2C	4921	G(D)	C(H)	C(H)	C(H)
3C	5661	T(K)	C(K)	C(K)	C(K)
3C	5967	C(S)	C(S)	C(S)	T(S)
3D	6667	C(P)	T(S)	T(S)	T(S)
3D	6669	T(P)	T(S)	T(S)	C(S)
3D	6904	G(A)	G(A)	T(S)	T(S)
3D	7152	C(I)	T(I)	T(I)	T(I)
3D	7341	G(A)	G(A)	G(A)	A(A)

[a] indicated the gene in which the points mutant located.
[b] indicated the position of the points of mutant in the genome.
[c] the bracket indicated the amino acid sequences encoded by the nucleotide sequences out of the bracket.

tivated P40 could protect ducks from the attack of Du/CH/LBJ/090809 in vaccine-challenge test, indicating that the two amino acid changes in VP1 do not affect the neutralization of the virus. Mutants in 2C and 3D proteins did not exist in the conserved motif. Proteins, such as VP3, 2A1, 2A2, 2B, 3A, 3B and 3C, had no amino acid changed during the passaging, which might suggest that these proteins might have less relationship to the pathogenicity and attenuation of DHV-1 viruses.

Generally, results of vaccine-challenge assay and the genomic sequence alignments indicated the antigenicity of the attenuated P40 might be similar to that of Du/CH/LBJ/090809. No functional descriptions were reported about the amino acids in the detected 9 points mutants in P40. We proposed that these mutations might be of great interest for investigation of the pathogenicity of DHV-1. There is no clue to say that the mutations we detected were the cause of the attenuation of the virus. However, we speculate that the attenuation might come from the change of the host and the total functions of all the mutations in amino acids.

Acknowledgements

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