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RESEARCH ARTICLE

Epidemiological Investigation of Duck Hepatitis A Virus (DHAV) Isolated from Sichuan Basin by RT-PCR Disclose the Existence of Mixed Infection and the Feasibility of DHAV-A Evolved from C80 Strain

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ABSTRACT

Thirty-five DHAV field isolates from suspected ducklings in Sichuan Basin in 2002-2010 were RT-PCR confirmed by sequencing their VP1 gene. Results demonstrated that DHAV-A (22) and DHAV-C (13) were dominant types. Furthermore, DHAV-A, DHAV-C infection and their co-infection still exists to some degrees. Gene analysis of VP1 disclosed that there are not obvious differences in nucleotides similarity and amino acid mutation except the V_{160} and $G/N/D_{187}$ for reference strain was changed as G_{160} and S_{187} for DHAV-A field strains and these two amino acid sites can be afterwards used as a beacon of molecular epidemiological investigation. Besides, all DHAV-A isolates possess nearest genetic relationship with reference strain C80 which was also previously isolated from China and used as vaccine strain. The feasibility that some variant strains were evolved from C80 strain may not be excluded.

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INTRODUCTION

Duck hepatitis virus (DHV) is a highly fatal and rapidly spreading viral of young ducklings, which primarily cause hepatitis (Gough *et al*., 1985). Till now, three different serotypes (DHV1, 2 and 3) have been historically found to be associated with the pathologic change in infected ducklings. Recently, several DHV-1 strains with unique characteristics are classified as a new member of Picornaviridae family (Ding *et al*, 2007; Kim *et al*., 2007). But, DHV-2 and DHV-3 are classified as a member of the genus for avian astrovirus (Tseng *et al*., 2007; Tseng and Tsai, 2007a) because they are more closely to members of Astroviridae. With the passage of time, two new serotypes carrying typical gene structure of DHV-1 but no cross-neutralization reactions were reported in Taiwan (Tseng and Tsai, 2007b) and in Korea (Kim *et al*., 2007a), respectively. The new viruses can also cause high mortality to ducklings with typical pathologic damage in liver similar to hepatitis. Totally, three serotypes of picornaviruses, and two kinds of astrovirus can cause duck viral hepatitis (Fu *et al*., 2009). To avoid confusions, duck hepatitis A virus (DHAV) in terminology was correspondingly used. That's to say, DHAV was divided into three serotypes including DHAV-A (formerly named as DHV-1), DHAV-B (isolated in Taiwan), and DHAV-C (isolated in South Korea).

Since DVH had been occurred in China, the main serotypes are composed of DHAV-A and DHAV-C (Wang *et al*., 2008). Practically, intensive application of attenuated vaccine or therapeutics antibodies also secured healthy development of waterfowl breeding. Unfortunately, such mentioned solutions were no longer validated because DHAV variant strain (Gao *et al*., 2012) or highly virulent strain (field isolate) (Xin Jin *et al*., 2008) in several regions was successively reported. Compared with the attenuated and tissue-adapted isolates, field isolates exhibit genetic diversity that there were two consistent amino acid substitutions ($E_{129}\rightarrow V_{129}$ and $A_{142}\rightarrow S_{142}$) between the field isolates and the tissue-adapted ones and six consistent differences for four attenuated Chinese isolates from the field isolates ($S_{181}\rightarrow L_{181}$, $H_{183}K_{184}\rightarrow R_{183}G_{184}$, $N_{193}\rightarrow D_{193}$, $E_{205}\rightarrow K_{205}$, $R_{217}\rightarrow K_{217}$, $N_{235}\rightarrow D_{235}$) in carboxyl terminal region (Liu *et al*., 2008b). Mixed infection by multiple serotypes is uncommon (Fu *et al*., 2008). Therefore, performing epidemiological investigation of DHAV extremely contributes to its effective control by vaccine development of or therapeutic antibody.

DHAV has three kinds of virion proteins (VP0, VP1 and VP3). The VP1, which locates in the surface of capsid with multiple T lymphocyte antigen sites and epitopes, can induce animal to produce neutralization antibodies. VP1 gene was probably the major virulent determinant (Xin Jin *et al*., 2008) and highly conserved within serotype, having more than 91% sequence homology at nucleotide level and 95% at amino acid level. In contrast, sequence variation among strains with different genotypes is considerable high, ranged from 28% to 34% at nucleotide level or 22% to 31% at amino acid level (Wang *et al*., 2008). Hence, VP1 gene was mainly used as target gene for molecular epidemiological investigation (Liu *et al*., 2008a). In this study, to get more insight into the relationship between genetic diversity and epidemiology status of DHVA in Sichuan Basin, we collected clinic samples from 2002 and 2010 and amplified their VP1 gene by RT-PCR. The gene was further cloned, and sequenced. Phylogenetic relationships between strains and serotypes were next analyzed. The etiology of DVAH was also explored and discussed.

MATERIALS AND METHODS

Samples were obtained from ducklings with typical clinic symptoms and pathologic changes in liver. Collected samples were immediately freeze at -70°C until use. Liver were grind in liquid nitrogen and resuspended in physiological saline with 20% ratio (w/v). The suspensions were then thawed three times. After centrifuged (12,000 g for 10 min) it and discarded the debris, the supernatants were finally used as RNA extraction template. The viral genomic RNAs were extracted from 200μL supernatant using MiniBEST Viral RNA/DNA Extraction Kit Ver.4.0 (TaKaRa Biotechnology, Dalian, China) according to manufacturer's instruction.

Virus strains and their accession numbers of gene in GenBank are listed in table 1. RT primers were six random bases. RT-PCR was performed according to manufacturer's (TaKaRa, China) instruction. PCR primers were designed by GeneFisher (2.0 version) and synthesized by Invitrogen (China). Primer Af (forward, 5' GGTGATTCTAACCAGTTAGG 3', nt 1-20) and Ar (reverse, 5'TTCAATTTCCAGATTGAGTTC 3', nt 694-714) aims to amplify a 714bp fragment (primers' position is referred to DHAV-A strain AV2111 VP1 protein gene -EF442073). Primer Bf (forward, 5'GGGGACACTAAC CAGCTTGGTG3', nt 2133-2154) and Br (reverse, 5'GATGGAGCTCAAACGCCAGGG3', nt 2815-2835) aims to amplify a 703bp fragment (primer's positions is referred to DHAV-B strain 90D genome -EF067924). Primer Cf (forward, 5' GGTGATTCCAATCAGCTTGG TGACG 3' nt 2132-2156) and Cr (reverse, 5'TTCCAAA TGGAGCTCAAAGGCAAG 3', nt 2822-2845) aims to amplify a 714bp fragment (primers positions is referred to DHAV-C strain AP-03337 genome -DQ256132). PCR was performed in 200μL thin walled PCR tube. The final 25 μL reaction volume with reagents: 2 μL template, 2.5 μL buffer, 1.25 U TaqDNA (TaKaRa), 5 mM $MgCL_2$, 0.2 mM of each dNTPs and 1 μM of forward and reverse primers. The reaction conditions were as follows: 94°C for 2 min, and then 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 50s and with a final step of 72°C for 5 min. DHV-2 and DHV-3 genes were detected by RT-PCR as described in literature (Todd *et al*., 2009).

The final PCR products were electrophoresed on a 1.2% agarose gel stained with 5μL/100mL Gold view. The purified fragment was ligated into pMD18-T Vector (TaKaRa, Dalian, china). 10μL ligation DNA product was transformed *Escherichia coli JM109* for later competent and white colonies screen. The target with desired insert was selected by colony PCR using previous described conditions. The screened bacterial colonies were cultured in LB broth (100 μg/mL ampicillin) and inserted DNA fragment was sequenced (Shanghai Songon, China).

VP1 gene sequences of other DHAV isolates were retrieved from GenBank (Table 1). Sequence was analyzed by DNAstar (DNASTAR Inc.) and Bioedit program. Based on the amino acid sequence of VP1 from DHAV-A or DHAC-C strains, a phylogenetic tree was constructed by the MEGA 4 Neighbor-Joining method (Tamura *et al*., 2007).

Table 1: Virus strains used in the analyses and their accession numbers in GenBank

DHAV-A strain	Origin, collection time	Accession No.
DHAV-A strain H	UK, vaccine strain	DQ249300
DHAV-A strain 5886	USA, vaccine strain	DQ249301
DHAV-A strain S	China: Beijing	EF417871
DHAV-A strain ZJ	southeast China	EF382778
DHAV-A strain ZI07-1	China: Zhejiang	EF502168
DHAV-A strain HSS	South Korea, 1995	DQ812092
DHAV-A strain XZ	China: XuZhou	EF653378
DHAV-A strain C-XNH	China: TianJin	EU621872
DHAV-A strain GHZ04	China: Guangdong, Huizhou, 2004	FJ496339
DHAV-A strain FFZ05	China: Fujian, Fuzhou, 2005	FJ496340
DHAV-A strain GL08	Vietnam: 2008	JF925122
DHAV-A strain 03D	Taiwan, field isolate	DQ249299
DHAV-A strain C80	China, attenuated isolate	DQ864514
DHAV-A strain SGO1	China: Shandong	JN225460
DHAV-A strain AP-03337	South Korea, 2003-4	DQ256132
DHAV-A strain GD1	China: Guangdong	EU289393
DHAV-A strain FS	China: Foshan; Mar-2008	EU877916
DHAV-A strain BM	Vietnam: 2009	JF925119
DHAV-A strain 1v	China: Shandong	GU250782
DHAV-A strain C-YCZ	China: 20-Jul-2005	FJ626672

RESULTS

PCR results of collected typical isolates were illustrated in Fig. 1 and detailed data were listed in Table 1. RT-PCR products profiles of 18 samples in 2002 were DHAV-A positive (8), DHAV-C positive (6) and co-infection of DHAV-A and DHAV-C (4). The 714 bp within vp1 gene of 22 DHAV-A and 13 DHAV-C field isolates were sequenced and compared between or among different strains. Sequence analysis revealed that these DHAV-A isolates shared 98.9-100% nucleotide and 97.5-100% amino acid sequence identities, and the DHAV-C isolates shared 91.6-99.7% nucleotide, 92.4-100% amino acid sequence identities. Molecular analysis further showed the field isolates and the reference strains are inherently being systematic differences. Except strain H (DQ249300) and FFZ05 (FJ496340), all field isolates and reference strains belonging to DHAV-A serotype can be grouped into two lineages. The genetic distances of the DHAV-A field isolates compared with reference strains were larger than that of the isolates. The C80 strain,

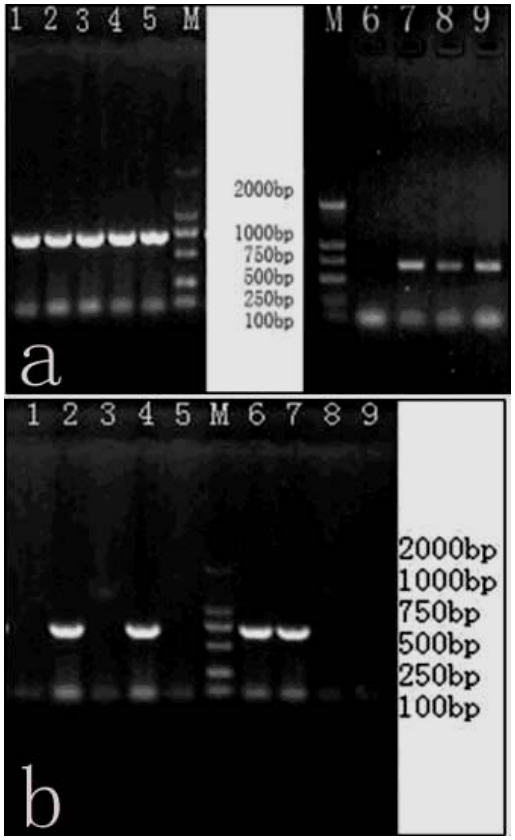

Fig. 1: Agarose gel electrophoresis of amplified PCR fragments of VP1 gene for DHAV field isolates. (a) PCR results of VP1 gene for DHAV-A. (b) PCR results of VP1 gene for DHAV-C. M: molecular mass markers 2000, 1000,750, 500, 250, 100 (bp). 1) Field isolate from Yongchuan (2002); 2) Field isolate from Dazu (2002); 3) Field isolate from Rongchuang (2002); 4) Field isolate from Mianyang (2002); 5) Field isolate from Xichang (2002), 6) Field isolate from Yongchuan (2010); 7) Field isolate from Huayang (2010); 8) Field isolate from Meishan (2010) and 9) Field isolate from Bishan (2010).

attenuated isolate, was most analog to the isolates from Sichuan Basin (Fig. 2). The 18 DHAV-C sequences were clustered into two major genogroups, namely, G1 and G2 (Fig. 3). G1 existed in all strains except for AP-03337 (DQ256132). It also contained a distinctive subgroup (SG2) for two field isolates (DZa, MYc), which, isolated in 2002 from a DHAV-A and DHAV-C co-infection duckling, had four amino acid substitutions compared with other strains ($V_{45}\rightarrow A_{45,}$ $H_{195}\rightarrow R_{195,}$ $K_{214}\rightarrow R_{214,}$ $H/L_{219}\rightarrow C_{219}$). The amino acids ($F_{35}$, R_{61}, T_{97}, S_{123}, P_{183}, G_{188} and L_{189}) completely consist with G2 and the amino acids (L_{54}, V_{160}, P_{178}, L_{185}, L_{186} and T_{191}) also with SG1 (Fig. 4). Sequence alignments also demonstrated there have two amino acid substitutions between the reference and the isolates ($V_{160}\rightarrow G_{160}$, $G/N/D_{187}\rightarrow S_{187}$) (Fig. 5).

DISCUSSION

DVH in China was previously controlled by attenuated vaccine made from traditional DHAV-A C80 strain in Sichuan Basin. Recently, some new cases were sporadically reported due to the existence of variant strain (Liu *et al*., 2011) or co-infection by DHAV-C and DHAV-A in ducklings or geese confirmed by RT-PCR

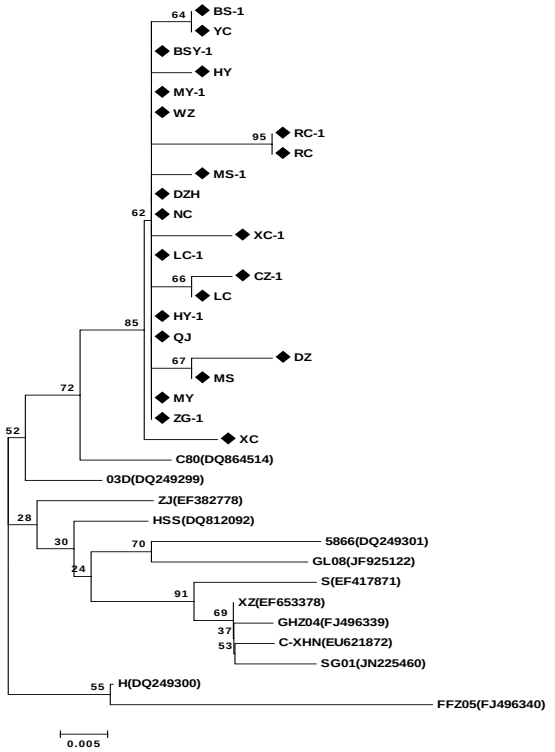

Fig. 2: Phylogenetic analysis of amino acid sequences of VP1 from DHAV-A isolate. Twenty-two field isolates in the present study are marked with ♦. Phylogenetic tree was constructed using neighbor-joining method by MEGA 4.0, and bootstrap value was calculated from 1,000 trees with equal input substitution model for amino acid sequences. The scale bar represents substitutions per site.

Fig. 3: Phylogenetic analysis of amino acid sequences of VP1 from DHAV-C isolate. The thirteen field strains in the present study are marked with ▲. Phylogenetic tree was constructed using neighbor-joining method by MEGA 4.0. and bootstrap value was calculated from 1,000 trees with equal input substitution model for amino acid sequences. The scale bar represents substitutions per site.

(Kim *et al*., 2008). The results indicate that DHAV-A, DHAV-C, and their combined infection from 2002 always exist among some ducklings in Sichuan Basin. This basically chimed in with the previous reports (Liu *et al*., 2011). Furthermore, positive rate of DHAV-C and co-infection in 2010 was obvious lower than that in 2002, which can be explained by intensive application of self-made tissue/inactivated vaccine. But, 12 samples in 2010 were DHAV-A (9), DHAV-C (2) and co-infection of DHAV-A and DHAV-C (1), respectively. DHAV-B, DHV-2 and DHV-3 were all not detected from

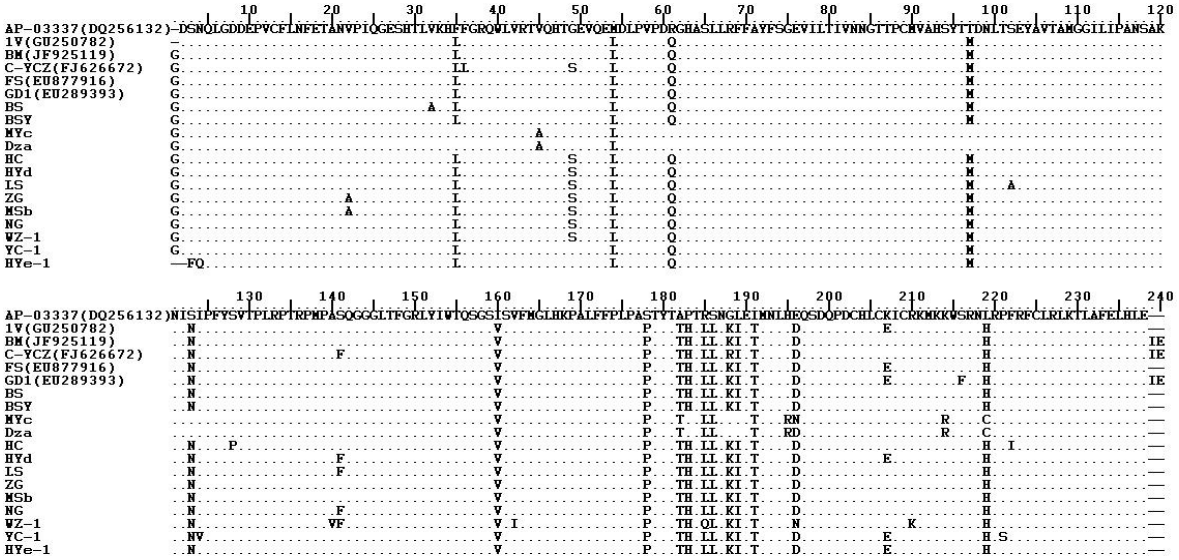

Fig. 4: Amino acid sequence alignment of the VP1 protein of DHAV-C isolate. The last thirteen lines are the field isolates from Sichuan Basin of China determined in this study. Residues are only shown the differences from the top line (reference strain AP-03337). The variable positions of isolate MYc and Dza were discussed in the text.

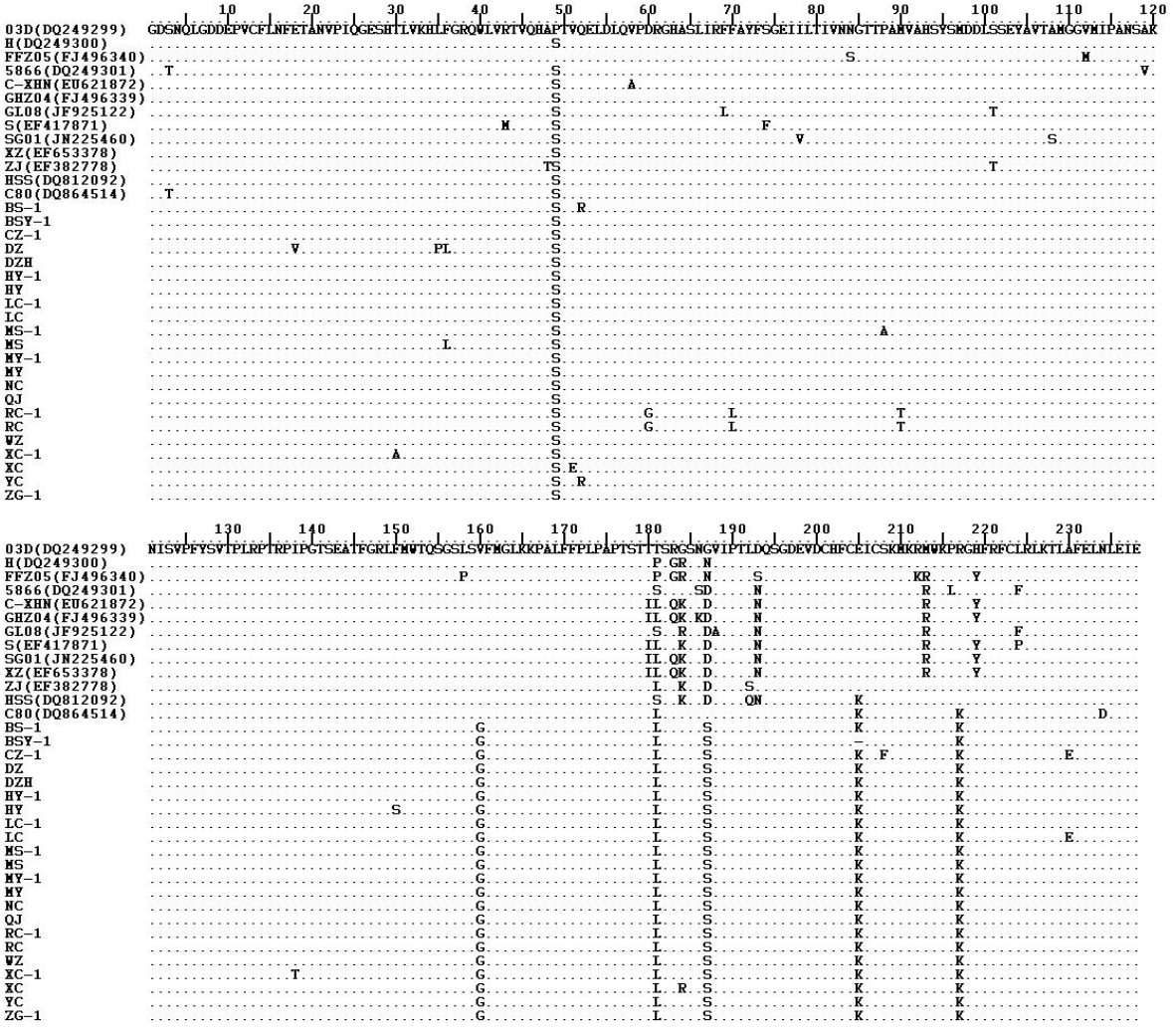

Fig. 5: Amino acid sequence alignment of the VP1 protein of DHAV-C isolates. The twenty-two lines are the field isolates from Sichuan Basin of China determined in this study. Residues are only shown differences from the top line (reference strain 03D).

these suspected samples. It means that DVH for susceptible ducklings is not effective controlled even though attenuated vaccine or therapeutic antibodies were intensively and widely used in Sichuan Basin. On the above grounds, SG2 strain seems to have evolved from SG1 and G2 strain.

There were two amino acid substitutions ($E_{129}\rightarrow V_{129}$ and $A_{142}\rightarrow S_{142}$) between field isolates and tissue-adapted ones, meanwhile four attenuated Chinese isolates showed six consistent differences from the field isolates ($S_{181}\rightarrow L_{181}$, $H_{183}K_{184}\rightarrow R_{183}G_{184}$, $N_{193}\rightarrow D_{193}$, $E_{205}\rightarrow K_{205}$, $R_{217}\rightarrow K_{217}$, $N_{235}\rightarrow D_{235}$)(Liu *et al*., 2008a). Early research disclosed that the carboxyl terminal region of DHAV was variable. In this study, the field isolates, which are not tissue –adapted ones, have amino acids (V_{129}, S_{142}, L_{181}, $R_{183}G_{184}$, D_{193}, K_{205}, K_{217}, D_{235}), but, the result of animal experiment showed that all of them have virulence to ducklings associated with liver necrosis, hemorrhagic and high mortality (data not shown). All 22 DHAV-A isolates were not duck/chicken embryo adopted and possess nearest genetic relationship with C80 strain in similarity 98.3-98.9% on nucleotide level or 97.1-98.3% on amino acid level carried typical feature of adopted strain containing V_{129} and S_{142} sites. Part of them has capability of virulence to ducklings with typical symptoms and pathological damages validated by pathogenic determination (they carried L_{181}, R_{183} G_{184}, D_{193}, K_{205}, K_{217}, D_{235} sites, Data not shown).

VP1 Gene analysis from 2002 and 2010 strains disclosed that there are not obvious differences in nucleotides similarity and amino acid mutation except the V_{160} and G/N/D_{187} in reference strain was changed to G_{160} and S_{187} in DHAV-A field strains. Therefore, we propose these two amino acid sites can be used as a beacon of molecular epidemiology investigation in Sichuan Basin. Thus, the feasibility that the former was evolved from the latter may not be excluded.

Conclusion: Thirty-five DHAV field isolates from suspected ducklings in Sichuan Basin from 2002 and 2010 were RT-PCR confirmed by sequencing their VP1 gene. Nucleotides analysis showed that DHAV-A, DHAV-C infection or co-infection were all existed. Gene analysis of VP1 disclosed that there are not obvious differences in nucleotides similarity and amino acid mutation except that the V_{160} and G/N/D_{187} in reference strain was changed as G_{160} and S_{187} in DHAV-A field strains, and these two amino acid sites can be used as the beacon of molecular epidemiology investigation. All DHAV-A isolates possess nearest genetic relationship with C80 strain in similarity 98.3-98.9% on nucleotide level or 97.1-98.3% on amino acid level. Part of them has the capability of virulence to ducklings with typical symptoms and pathological damages validated by pathogenic determination. The feasibility that the DHAV-A prevalent strain was evolved from the attenuated strain might not be excluded.

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REFERENCES

Ding C and D Zhang, 2007. Molecular analysis of duck hepatitis virus type 1. Virol, 361: 9-17.

Fu Y, M Pan, X Wang, Y Xu, X Xie, N Knowles, H Yang and D Zhang, 2009. Complete sequence of a duck astrovirus associated with fatal hepatitis in ducklings. J Gen Vir 90:1104-1108.

Fu, Y, M Pan, X Wang, Y Xu, H Yang and D Zhang, 2008. Molecular detection and typing of duck hepatitis A virus directly from clinical specimens. Vet Microbiol, 131: 247-257.

Gao J, J Chen and X Si, 2012. Genetic Variation of the VP1 Gene of the Virulent Duck Hepatitis A Virus Type 1 (DHAV-1) Isolates in Shandong Province of China. Virol Sin, 27: 248-253.

Gough RE, ED Borland, IF Keymer and JC Stuart, 1985. An outbreak of duck hepatitis type II in commercial ducks. Avian Pathol, 14: 227-236.

Kim M, Y Kwon, S Joh, S Kim, C Tolf, J Kim, H Sung, A Lindberg and J Kwon, 2007. Recent Korean isolates of duck hepatitis virus reveal the presence of a new geno- and serotype when compared to duck hepatitis virus type 1 type strains.Arch Virol, 152: 2059-2072.

Kim, M, Y Kwon, S Joh, J Kwon and A Lindberg, 2008. Differential diagnosis between type-specific duck hepatitis virus type 1 (DHV-1) and recent Korean DHV-1-like isolates using a multiplex polymerase chain reaction. Avian Pathol, 37: 171-177.

Liu G, Ni Wang, T Yun, B Yu, J Huang and J Chen, 2008. Genetic diversity of the VP1 gene of duck hepatitis virus type 1 (DHV-1) isolates from southeast China is related to isolate attenuation. Virus Res, 137: 137-141.

Liu G, F Wang, Z Ni, T Yun, B Yu, J Huang and J Chen, 2008b. Genetic diversity of the VP1 gene of duck hepatitis virus type 1 (DHV-1) isolates from southeast China is related to isolate attenuation.Virus Res, 137: 137-141.

Liu G, E Yángüez, Z Chen and C Li, 2011. The duck hepatitis virus 5'-UTR possesses HCVlike IRES activity that is independent of eIF4F complex and modulated by downstream coding sequences. Virol J, 8: 147.

Tamura K, J Dudley, M Nei and S Kumar, 2007. MEGA 4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol, 24: 1596-1599.

Todd D, V Smyth and N Ball, 2009. Identification of chicken enterovirus-like viruses, duck hepatitis virus (DHV) type 2 and DHV type 3 as astroviruses. Avian Pathol, 38: 21-39.

Tseng C, 2007. Molecular characterization of a new serotype of duck hepatitis virus. Virus Res, 126: 19-31.

Tseng CH and HJ Tsai, 2007. Molecular analysis of duck hepatitis virus type 1 indicates that it should be assigned to a new genus. Virus Res, 123: 190-203.

Wang Y, C Li and Z Chen, 2012. Complete genome comparison of duck hepatitis virus type 1 parental and attenuated strains. Virus Genes, 45: 398-401.

Xin J, Z Wei, W Zhang, C Gu, G Cheng and X Hu, 2008. Identification and molecular analysis of the highly pathogenic duck hepatitis virus type 1 in Hubei province of China. Res Vet Sci, 85: 595-598.