Identification and molecular analysis of the highly pathogenic duck hepatitis virus type 1 in Hubei province of China

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Abstract

Duck hepatitis virus types 1 (DHV-1) JX strain was isolated from infected ducklings with clinical symptoms from Hubei province of China. These isolated strains showed high pathogenicity to both duck embryo and duckling in Duck embryo neutralization assay and animal infection experiment. The complete genome of JX strain was sequenced. Comparative genome analysis with other available strains in GenBank indicated that JX strain shared 94–99% similarity at the nucleotide level and 95–99% at amino acid level with other DHV-1 strains. Sequence results showed that mutations of nucleotide and amino acid were mainly distributed in VP1 genes. Our result implied that the VP1 probably was the major virulent determinant of DHV-1. In addition, 13 DHV-1 strains from different area were analyzed in phylogeny and they can be grouped into four distinct lineages. The new-identified JX strain was grouped into one lineage with A66 and C80 strains, which were also isolated from China.

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1. Introduction

Duck virus hepatitis (DVH) is a fatal and rapidly spreading disease of young ducklings (Woolcock, 2003). The major pathologic change in infected ducklings is hepatitis. Three different viruses, duck hepatitis virus (DHV) types 1, 2 and 3, have been found to be associated with these symptoms. Among them, DHV-1 is the most virulent, which can cause mortality higher than 80% in ducklings younger than three weeks. It has a worldwide distribution and threatens to all duck-growing farms (McNulty, 2001; Woolcock, 2003). DHV-1 was previously classified as an enterovirus, primarily based on the observed morphology and physicochemical properties of the virion (Kim et al., 2006). Recently, the genome sequences of several DHV-1 strains were determined. Comparative sequence analyses showed that the DHV-1 is classified as a member of the Picornaviridae family. However, it possesses three unique in-tandem 2A genes. 2A1 protein is an aphthovirus-like 2A protein and 2A3 protein is a human parechovirus-like 2A protein but 2A2 protein is not related to any known picornavirus proteins. Phylogenetic and evolutionary analysis indicated that DHV-1 was closer to members of the genus parechovirus than to other picornaviruses (Tseng et al., 2007). It has been proposed that it could be assigned as a new genus in the Picornaviridae family (Ding and Zhang, 2007). In addition, the 3’ UTR of DHV-1 is the largest fragment among the picornaviruses and its capsid polypeptide VP0 is not proteolytically cleaved into VP4 and VP2.

Up to now, 13 complete genomic sequences of DHV-1 have been deposited in GenBank, including attenuated vaccine strains and virulent field strains. The relationship of molecular sequences and virulence was not understood well and the virulent determinants were unknown yet. In this study, JX, a virulent field strain of DHV-1 was identified and its pathogenicity to both duck embryo and duckling was tested. Moreover, its genomic sequence was analyzed and compared with other strains in Genebank.
and showed high similarity in nucleotide and amino acid sequences. The 13 strains were grouped into four lineages and JX is grouped into one lineage with other two strains isolated in China. Based on mutation distribution analysis, it was proposed that VP1 gene may have great influence on the pathogenicity of DHV-1.

2. Materials and methods

2.1. Ducklings, embryonic duck eggs, and antisera

One-day-old ducklings and ten-day-old embryonic duck eggs were purchased from Dadi breeding duck farm (Wuhan, China) which were free from DHV-1. DHV-1 antisera were purchased from China Institution of Veterinary Drug Control (Beijing, China). The neutralization titer of DHV-1 antisera was 1:320.

2.2. Case history and virus isolation

Suspicious DHV-1 infections were found in five-day-old ducklings in Wuhan, China at March 2007. Ducklings (2000) of a farm were affected, and more than 800 sick ducklings died in three days. The clinical signs included lethargy, weakness, lateral recumbency, epileptic seizures, and opisthotonos. The sick ducklings died quickly after presentation of clinical signs. Gross lesions were restricted to the liver, which were swollen, and presents multiple punctate hemorrhages or coalescing hemorrhages. The liver samples of some sick ducklings were collected for virus isolation.

The liver samples were homogenized with sterile 0.01 M phosphate-buffered saline (PBS) (pH 7.2). After freezing and thawing for three times, the liver homogenate was centrifuged for 20 min at 8000 g. Antibiotics (penicillin 10,000 IU/ml and streptomycin 10,000 IU/ml) were added to the supernatant, and then the samples were incubated at 37 °C for up to 1 h. After incubating, the supernatant were filtered with a 220 nm filter. The filtrates were inoculated into 11-day-old embryonated duck eggs through the allantoic route, 0.2 mL per egg. Embryonic duck eggs were incubated at 37 °C for up to 96 h, and then allantoic liquid of dead embryos was collected under routine conditions. The virion RNA was extracted from allantoic liquid of dead duck embryos which were from eggs inoculated with the DVH1 JX strain by the Viral RNA Mini Kit (Takara, China) according to the manufacturer’s instructions, and was used in the following studies.

2.5. Isolation and purification of DHV-1 RNA

The RT-PCR primers were designed based on the completed genome sequence of DHV-1 (GenBank accession number NC008250). Primer sequences were listed in Table 1. The amplified sequences spanned the whole open reading frame (ORF) of DHV-1 genome. RT-PCR was performed utilizing one step RNA PCR kit (TaKaRa, China). The RT-PCR condition was 50 °C for 30 min, 94 °C for 2 min, and then 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and with a final step of 72 °C for 5 min. The PCR-products were run on a 1% agarose gel and purified with DNA Fragment Purification kit (Toyobo, Japan). The purified PCR-products were TA-cloned into pMD18-T vector (TaKaRa, China), and then sent to commercial service for sequencing (Invitrogen Biotechnology Corporation). The pair-wise sequence identities among the nucleotide and amino acid sequences of the JX strain with other DHV-1 strains were determined by using BLAST method.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) and sequencing of DHV-1

Phylogenetic analysis was carried out by analyzing the data obtained in present study with those of other sequences of DHV-1 from the GenBank database. Sequence alignments were generated by CLUSTAL X (Version 1.83; Thompson et al., 1997). Phylogenetic trees were constructed from the aligned 13 polypeptide amino acid sequences of DHV-1 by MEGA Version 3.1 (Kumar et al., 2004) with 1000 bootstrap replicates.
3. Results

3.1. Virus isolation

The isolated virus was shown to induce the pathological symptoms in infected embryos, including dwarfing, hyperaemia of body surface, swollen and hemorrhages of liver, meningorrhagia and death of embryos between 48 and 96 h post-inoculation. The allantoic fluid of death embryo showed light green color.

3.2. Chicken embryo cross-neutralization assays

Eight days after inoculation, the eggs were opened and examined for the lesions typical for the DHV infection. Embryos inoculated with virus–serum mixtures were all survived apart from one embryo inoculated of 160°C2 virus–serum mixtures. Blank controls were all surviving and the no serum controls all died 72–96 h post-inoculation. All of the dead embryos presented similar symptoms including hyperaemia of body surface, swollen and hemorrhages.

3.3. Animal experiments

Four ducklings inoculated with allantoic liquid containing DHV-1 viruses died 48 h post-inoculation. The clinical symptoms include depressed, wing drooping, anorexia. All died ducklings showed opisthotonus. Gross lesions tend to be restricted to the liver, which is swollen, fragile, and presents multiple punctate hemorrhages. Spleen and kidney also presented various levels swollen. The surface of spleen showed multiple punctate hemorrhages. Ducklings inoculated with 0.9% NaCl were all surviving and showed no obvious clinical symptoms and gross lesions.

3.4. Features of the DHV-1 genome and deduced polypeptide sequence

All nine DHV-1 fragments obtained in RT-PCR were sequenced and aligned. The nearly complete genome sequence of DHV-1 JX strain contains 7582 nucleotides, and encodes 2249 amino acids. The nucleotide sequences determined in this study are available from GenBank under accession number EF093502. It shared 99% similarity at the nucleotide level with the C80 and A66 strains genome, and 94–96% similarity at the nucleotide level with that of other DHV-1 strains listed in GenBank. The results of amino acid sequence alignments of DHV-1 showed that the main differences at amino acid level of DHV-1 existed in VP1.

3.5. Phylogenetic analysis

Phylogenetic analysis was performed using the full amino acid sequence of DHV-1 (Table 2). Phylogenetic tree revealed that there were four distinct groups. JX, A66 and C80 strains were grouped into a branch with high bootstrap supporting value. E53, S and ZJ strains formed another group. Strains (5886) were grouped into a single branch and 008250, O3D, H, DRL-62, R85952, HP-1 strains belonged to the largest branch (Fig. 1).

4. Discussion

Most recently, the genome sequences of five DHV-1 strains were determined (Kim et al., 2006; Tseng et al., 2007), which enables us to compare them at the nucleic acid and protein levels. Comparative sequence analyses showed that they possessed a typical picornavirus genome organization apart from the unique possession of three in-tandem 2A genes in DHV-1. The 2A1 protein of DHV-1 is an aphthovirus-like 2A protein while the 2A2 protein is not related to any known picornavirus protein. The 2A3 protein is a human parechovirus-like 2A protein. In addition, several other features including the 3’ UTR and amino acid sequence identities were found to be unique in the DHV-1 genome.
When we investigated the virulence of several DHV-1 strains isolated by our laboratory, it was found that different strain possess distinct virulence to duck embryos and ducklings (data not shown). The virulent determinant of DHV-1 was far from clear. In this study, through data analysis, we found that 13 DHV-1 strains from different area shared high homology at amino acid level except the high variation of VP1. A hypothesis emerged that the changes in amino acid of the VP1 gene may have influence on the pathogenicity of DHV-1. Test of this will need further studies.

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References


