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Synonymous codon usage of the VP2 gene of a very virulent infectious bursal disease virus isolate serial passaged in chicken embryos

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ABSTRACT

Three very virulent infectious bursal disease virus (vvIBDV) strains were isolated from a single farm and shown to be phylogenetically related to the vvIBDV isolate UK661. In this study, a comparative analysis of the synonymous codon usage in the hypervariable region of theVP2 (vVP2) gene of the vvIBDV strains was done on viruses serially passaged in chicken embryos. Sequencing demonstrated that codons change during the serial passage in the vVP2 gene of the viruses. Nine codon mutations resulted in amino acids changes. The amino acid changes were I256V, I296L 6in isolate XA1989, A222P, I242V, Q253H, I256V in isolate XA1998, and Q253H, I256V, I296L in isolate XA2004. Three of the nine amino acid changes occurred at residue 256. The codons of the amino acids A232, N233, I234, T269, T283 and H338 changed to the synonymous codons in XA1989 after the 16th passage, in XA1998 after the 24th passage and in XA2004 22nd passage viruses. These mutations change the key amino acid residues Q253H and I256V in the domains which are essential for its virulence, and the synonymous codons were observed compared to classical virulent IBDV. The results indicated that the codon changes during the serial passage comprised of synonymous codon usage in the vVP2 gene of IBDV, and this synonymous codon bias was correlated with pathotypes. The extent of synonymous codon usage bias in the IBDV-vVP2 gene maybe influence the gene expression level and secondary structure of protein as well as hydrophobicity, therefore the results provide useful perspectives for evolution and understanding of the pathogenesis of IBDV.

1. Introduction

Synonymous codons do not occur at equal frequencies. Viruses very often have quite biased preferences for some synonymous codons over other possible synonymous nucleotide triplets coding for the same amino acids. Studies on synonymous codons usage in viruses revealed that they vary between genes and even between the different parts of a gene (Zhao et al., 2008). In general, highly expressed genes have a strong preference for a subset of codons, while less expressed genes have a more uniform pattern of codon usage (Gouy and Gautier, 1982; Grosjean and Fiers, 1982). Synonymous codon substitutions lead to identical protein sequences. Remarkably, these alternative sequences give protein products with similar but different structures and functions (Adzhubei et al., 1996, 1998; Das et al., 2006; Oresic and Shalloway, 1998; Tsai et al., 2008). The present study unambiguously depict that the patterns of synonymous codon usage within a protein domain can change dramatically during the course of evolution to give rise to pathogenicity (Basak et al., 2009).

Infectious bursal disease virus (IBDV) is a double-stranded RNA virus in the *Birnaviridae* family. It is divided into attenuated, classical virulent, very virulent and antigenic variant strains, based on pathotype. The capsid of IBDV contains VP1, VP2, VP3, VP4 and VP5 structural proteins. (Mundt et al., 1995). Serotype-specific antigenic determinants inducing neutralizing antibody binding sites are located on VP2, whereas antigenic sites recognized by group-specific monoclonal antibodies are located on structural proteins, VP2 and VP3 (Snyder et al., 1988). The hypervariable region of VP2 (vVP2) is often the region of interest in molecular epidemiology and phylogenetic studies. Tracking IBDV using the vVP2 region that is subject to frequent mutations allows greater discrimination between genomes that are closely related (Levin et al., 1999).

In the vVP2, attenuated, classical virulent, antigenic variant and vvIBDV strains have their characteristic amino acid residues. The amino acids at the positions of P222, V242, V256, T270, I294, N299 and the sequences of the heptapeptide motif at position 326–332 are SWSARGS or SWSAKGS were observed in most attenuated and classical virulent IBDV strains (Jackwood and Sommer-Wagner, 2006; Rudd et al., 2002; Yamaguchi et al., 1996a,b, 1997). The amino acids A222, I242, I256, I294S, 299 and the heptapeptide motif SWSASGS were reported to be highly conserved among vvIBDV strains (Banda and Villegas, 2004; Hoque et al., 2001; Kasanga

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et al., 2007; Kong et al., 2004; Rudd et al., 2002). Although the sequences SWSASGS were common between antigenic variant and vvIBDV strains, the K249 and I286 were unique to the antigenic variant strain, and the amino acids A222, V242, V256 and L294 were reported to be conserved among antigenic variant IBDV strains (Jackwood and Sommer-Wagner, 2006; Kasanga et al., 2007).

Recently we reported on viruses isolated from a single farm during 1989 to 2008 where it was shown that all the isolates were derived from vvIBDV (Li et al., 2009). The purpose of this study was to assess the genetic variability among the field isolates of IBDV associated with different passages in the chicken embryo and to track the changes of the codon of the viral dsRNA from these strains, estimating the correlation of synonymous codon usage bias with pathotypes. To achieve these goals, we have examined the nucleotide sequences of the hypervariable region VP2 from IBDV during the serial passage in the chicken embryo.

2. Materials and Methods

2.1. Virus Strains and Infectivity Titrations

XA1989, XA1998 and XA2004 IBDV strains were isolated from field outbreaks in commercial flocks on one farm in Xinan County, Henan Province, PR China. Pathological examination of sick chickens showed severe gross lesions in the bursa of Fabricius and spleen, which are signs of IBDV infection. The bursas were collected aseptically into normal saline (w/v = 1:3). Each bursa was minced using scissors and passed through a steel mesh to obtain a homogeneous suspension. The homogenate was subjected to three freeze–thaw cycles. After centrifugation at 12,000 × g for 15 min at 4 °C, the supernatant was collected. Chicken embryo fibroblasts (CEF) were prepared according to standard procedures and grown as monolayers on plastic petri dishes (5 cm diameter) for infectivity titrations. The supernatant was diluted serially and infectivity titrations were performed as plaque assays (Nick et al., 1976).

2.2. SPF Eggs

Specific pathogen free (SPF) eggs were purchased from Nanjing Medical University, Nanjing, PR China.

2.3. Virus Inoculation to SPF Chicken Embryos

Three 9-day-old SPF-embryonated eggs were inoculated with 0.2 ml (1×10^6 PFU/ml) of each bursa suspension of the isolates XA1989, XA1998 and XA2004 via the chorioallantoic membrane route, respectively. The SPF-embryonated eggs were incubated at 37 °C for 96 h. Ninety-six hours post-inoculation, the harvested allantoic fluids of the isolates XA1989, XA1998 and XA2004 were passaged on the 9-day-old SPF-embryonated eggs. The viruses serially passaged 30 times were designated as the E01–E30 viruses, respectively. Serial passages of IBDV virus were collected and carried out to sequencing. Infectivity titres and Embryo lethal dose$_{50}$ (ELD$_{50}$) of the nucleotide sequence variable passaged viruses were detected. During passage, SPF-embryonated eggs were inoculated with the same amount of infectious virus in original isolates.

2.4. Extraction of Viral RNA

The bursas supernatant of the isolates XA1989, XA1998, XA2004 and the harvested allantoic fluids of their passaged viruses were digested with proteinase K (0.5 mg/ml) for 2 h at 50 °C, respectively. The mixture was extracted twice with phenol/chloroform/isoamylalcohol (25:24:1) and once with phenol/chloroform (25:24). The viral genome was precipitated with ethanol in the presence of sodium acetate (0.3 M final concentration, pH 5.6). Finally, the total RNA were dried, resuspended in 30 μl RNase-free water, and used for reverse transcription-PCR (RT-PCR).

2.5. Reverse Transcription-PCR

The RT-PCR assay was conducted using a primer pair that amplifies a 540 bp region of vVP2. This region of the genome was selected because it encodes the most highly variable sequence of the virus and has numerous strain specific genetic markers (Banda and Villegas, 2004; Hoque et al., 2001; Parede et al., 2003; Rudd et al., 2002). The forward primer was 5′-GCCGATGATTACCAATTCTCATC-3′ (located at 633–656) and the reverse primer was 5′-GCAAATCGGCCGTATTCTGTG-3′ (located at 1152–1172). The primers were designed based on the consensus sequences of vVP2 of classical virulent IBDV strain Harbin (GenBank accession: AF092171) and vvIBDV strain HK46 (AF051838), with only one nucleotide at the 5′-end of the forward primer different from that variant viruses Del-E (X54858). The primers were applicable to classical virulent and very virulent as well as antigenic variant IBDV strains. The RT-PCR reactions were conducted using the TakaRa RNA PCR kit (AMV) Ver.3.0 (TakaRa Bio Inc., Otsu, Japan) following the manufacturer's instructions. The reaction mix was incubated at 42 °C for 45 min. The template was amplified with 36 PCR cycles at 95 °C for 1.0 min, 56 °C for 45 s and 72 °C for 50 s. A 10 min extension at 72 °C was added at the end of PCR cycles.

2.6. Nucleotide Sequence Analysis

The RT-PCR products were purified from 1% agarose gel. They were sent to TakaRa Biotechnology (Dalian) Company (Dalian, China) for nucleotide sequencing. The nucleotide and deduced amino acid sequences were analyzed with the aid of Clustal X version 1.83 software. A phylogenetic tree was constructed using a DNA parsimony program and neighbor-joining methods based on the 540 bp sequence in the VP2 gene of original, passage IBDV isolates and reference IBDV strains, using a nucleotide alignment created in MEGA 4.1 software. The topological accuracy of the tree was estimated by the bootstrap method with 500 replicates. Reference IBDV strains including known antigenic variant strains Del-E (GenBank accession: X54858), Var-E (AF133904); P2 attenuated IBDV strain (X84034), classical cell culture-adapted Cu-1 (X16107); classical virulent IBDV strains JD1 (AF321055), Harbin strain (AF092171), B87 (DQ906921) and vvIBDV strains UK661 (X92760), KK1 (AF165150), HK46 (AF051838), UPM97/61 (AF247006) were used for comparison in both phylogenetic trees and nucleotide sequence analysis. The sequences of reference IBDV strains were obtained from GenBank.

2.7. Infectivity Titrations and ELD$_{50}$ Determination in SPF Chicken Embryos

The bursa suspension of the isolates XA1989, XA1998, XA2004 and their sequence variable passaged viruses infectivity titres were detected, and concentration of these virus were adjusted to 1×10^8 PFU/ml, then, 9-day-old SPF-embryonated eggs were inoculated with 0.4 ml (4×10^7 PFU/0.4 ml) of these virus in different dilution (10^{-3}–10^{-9}) via the chorioallantoic membrane route and were incubated at 37 °C for one week. The number of dead embryos was recorded every 6 h. Embryo lethal dose$_{50}$ (ELD$_{50}$) of the virus isolate was determined using embryonated eggs following the Reed and Muench method (Reed and Muench, 1938).

3. Results

3.1. Nucleotide Sequence Analysis of the vVP2 Gene in Original and Passaged Viruses

The sequences of the vVP2 (nucleotides 633–1172) of these viruses were determined every two passages until no nucleotide changes occurred in the following six passages in chicken embryos. Sequencing demonstrated that the codon changes during serial passages were comprised of synonymous codons in the vVP2 gene of the virus (Fig. 1). The nucleotide sequences of the initial isolates XA1989, XA1998 and XA2004 changed when they were passed in chicken embryos. The initial passaged viruses sequence was identical to the original virus. With the serial passage, the codon initial changed in XA1989 at the 6th passage, in XA1998 at the 8th passage and in XA2004 at the 4th passage. There were no obvious changes in XA1989 after 16th passages, in XA1998 after 24 and XA2004 after 22 passages. A phylogenetic tree based on the nucleotide sequences of the vVP2 (Fig. 2) showed that the homology of nucleotide with the P2 attenuated IBDV strain and classical cell culture-adapted IBDV strain Cu-1 are increased along with the number of passages in chicken embryos. Compared to the original isolates XA1989, XA1998 and XA2004, the vVP2 of their passaged viruses had nineteen synonymous codons substituted, but six synonymous codons were substituted at the same amino acids: A232 (GCT → GCC), N233 (AAT → AAC), I234 (ATC → ATT), T269 (ACT → ACA), T283 (ACG → ACA) and H338 (CAC → CAT). In addition, the synonymous codons of the amino acids at position A259 (GCT → GCC) and Q320 (CAG → CAA) were substituted in XA1998 and XA2004.

3.2. Amino Acid Sequence Analysis of the vVP2 in Original and Passaged Viruses

A phylogenetic tree based on the amino acid sequences of the vVP2 is shown in Fig. 3. The homology of amino acids with the P2 attenuated IBDV strain and classical cell culture-adapted IBDV strain Cu-1 increased with the number of passages through