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Full Length Research Paper

Molecular cloning and sequence analysis of VP6 gene of giant panda rotavirus strain CH-1

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Rotavirus (family *Reoviridae*) is the leading cause of severe gastroenteritis in human and animals worldwide. The genome of rotavirus comprises of 11 segments of dsRNA and encodes six structural proteins (VP1 to VP4, VP6 and VP7) and six non structural proteins (NSP1 to NSP6). VP6 is a group of antigen of rotavirus (according to the disparation of VP6, rotavirus is classified into seven groups: A to G), and the major structural protein of inner capsid particles (ICP), and also specific antigen of mucosa immunization that mediate specific immunological reaction. In this report, sequence analysis of VP6 gene of giant panda rotavirus was carried out. Full-length VP6 gene encoding for ICP of giant panda rotavirus was amplified by RT-PCR and the amplicons (1356 bp) were cloned and sequenced. Comparative sequence analysis revealed an open reading frame of 1194 nucleotides (nt) encoding a polypeptide of 397 amino acids (aa). Porcine and human rotaviruses VP6 were highly related giant panda rotavirus VP6 with sequence identity of 98.7 and 97% at the aa level, respectively. Further, they showed 62.9 to 95.1% sequence identity at the nt level with other species of rotavirus. Phylogenetic analysis also showed that giant panda rotavirus VP6 gene was closely related to porcine and human rotavirus. Together, these results may improve our understanding of the evolution, pathogenesis and functional studies of giant panda rotavirus, as well as contribute significantly to giant panda rotavirus research and possibly studies with other species rotaviruses.

Key words: Giant panda rotavirus, VP6 gene, molecular cloning, sequence analysis.

INTRODUCTION

The giant panda (*Ailuropoda melanoleuca*) is one of the world's most recognized and threatened animals (O'Brien et al., 1994; Peng et al., 2001). Currently giant pandas are restricted to the isolated Qinling, Minshan, Qionglai, Daxiangling, Xiaoxiangling and Liangshan mountains (Zhang and Wei, 2006).

Among all the diseases in the giant panda, diarrhoea disease is one of the most serious. Rotaviruses are known to be major causative agents of severe acute gastroenteritis in infants and young animals. Globally, rotavirus is responsible for enormous morbidity and is estimated to cause 114 million episodes of diarrhoea peryear (Grimwood and Buttery, 2007), and remain a major health problem worldwide. Once giant panda is infected by rotavirus, there is no sign before it falls ill; but suddenly apastia and intense disgorging appear, then, key feature of the disease such as water-like diarrhea,

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abdominal gaseous distention, diarrhoea protraction and so on appears, and it eventually die of multi-organ non-function (Wang et al., 2008).

Rotavirus is a triple-layered icosahedral protein capsid surrounding a genome of double-stranded (ds) RNA, of approximately 70 nm in diameter, belonging to the *Reoviridae* family. The rotavirus genome comprises 11 segments of dsRNA and encodes six structural and six non structural proteins. The most external capsid consists of the proteins VP4 and VP7. The intermediate capsid is constituted by VP6. VP6 plays several important roles in the replication cycle of rotavirus: (1) VP6 is a structural protein on the surface of the immature inner capsid particle (ICP) and assembles as 260 trimmers with a T = 13l icosahedral lattice (Roseto et al., 1979; Prasad et al., 1988; Yeager et al., 1990); (2) VP6 is a necessary component for the ICP to be transcriptionally active (Bican et al., 1982; Sandino et al.,1986; Both et al., 1994); (3) VP6 binds to a virally encoded glycoprotein receptor (NSP4, formerly NS28), which mediates the budding of the immature ICP into the endoplasmic reticulum (ER) where final maturation and assembly of the virus takes place (Meyer et al., 1989). VP6 is the subgroup-specific antigen for rotavirus (Kalian et al., 1981; Matsui et al., 1989), and IgA neutralizing antibodies directed against VP6 can protect against rotavirus infection (Burns et al., 1996).

In our research, we cloned and sequenced VP6 of giant panda rotavirus which was propagated with MA-104, and sequence analysis was carried out by bioinformatics software. The results from this study provide a basis for further functional analysis of this gene and some interesting data that may be beneficial to evaluate the outbreak of rotavirus in giant panda infection.

MATERIALS AND METHODS

Giant panda rotavirus strain CH-1, was isolated from Chengdu Research Base of Giant Panda Breeding, and preserved in the author's laboratory.

Primer design

Based on the cDNA sequences of genomic segments VP6 of rotavirus from GenBank (Accession No: GU188283), a pair of primers R1/F1 was designed and used to amplify the VP6 gene (1356 bp) of giant panda rotavirus strain CH-1. Primer sequences were as follows: R1, 5'-CGAATTCGGCTTTTAAACGAAGTCTTC-3' and F1, 5'-GCTCGAGGGTCACATCCTCTCACTA-3'. *Eco*RI and *Xho*I (underlined) sites were incorporated into the forward and reverse primers, respectively.

Preparation of virus and RNA extraction

The giant panda rotavirus strain CH-1 was propagated in MA-104 that were grown in Dulbecco's minimum essential medium (D-MEM, Gibco-BRL) supplemented with 10% FBS at 37°C. For virus infection, D-MEM supplemented with 1 µg/ml trypsin was used. Viral particles were harvested when the cytopathic effect reached 75%; the infected culture fluid was frozen and thawed three times. After centrifugation, the supernatant was stored at -70°C until use. Total viral genomic RNA was extracted with RNAiso reagent (TaKaRa, Dalian, China) according to the manufacturer's instruction: 400 µl of infected culture supernatant was mixed with 600 ml of RNAiso reagent. The mixture was mixed well and incubated for 5 min. After adding 120 µl of chloroform, the tubes were mixed by inversion and shaken vigorously for 15 s. The mixture was incubated for 5 min and centrifuged at 13,000 × *g* for 15 min at 4°C to separate it into two phases. The upper aqueous phase at which viral RNA remained was transferred to a fresh microcentrifuge tube, and the equivalent volume of isopropyl alcohol was added to the sample. The tubes were mixed by inversion and incubated for 15 min at room temperature. To precipitate viral RNA, the mixture was centrifuged at 13,000 × *g* for 15 min at 4°C, and the RNA pellets were washed with 1,000 ml of 75% ethanol. The obtained RNA pellet was dried for 5 min and dissolved in 40 µl of sterile diethylpyrocarbonate (DEPC)-treated water. The RNA was purified from culture supernatant according to the instruction of RNAiso PLus. In brief, 600 µl RNAiso PLus solution was added to 400 µl culture supernatant, homogenized, and stood at room temperature for 5 min. Then, 120 µl chloroform was added and the tube was placed in a shaker for 5 min, stood at room temperature for 5 min, and centrifuged at 13000 *g* for 15 min at 4°C. The clean supernatant was transferred into a new tube, equal volume of isopropyl alcohol was added and gently mixed by inverting and rotating the tube several times, then the tube was stood at room temperature for 10 min, centrifuged at 13000 *g* for 10 min at 4°C, 1 ml of 75% alcohol was added to the tube to wash the precipitation, centrifuged at 13000 *g* for 5 min at 4°C, and clean supernatant was discarded while the precipitation was left and dried. 40 µl of TE buffer [10mM Tris-HCl (pH 7.4), 1mM EDTA] was added to dissolve the precipitation and stored at -70°C for future use.

RT-PCR and sequencing of giant panda rotavirus strain CH-1 VP6 gene

Reverse transcription was carried out by PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instruction: 37°C for 15 min, 85°C for 5 s using 7 µl of RNA, 2 µl 5×PrimeScript buffer, 0.5 µl random primer and 0.5 µl PrimeScript RT Enzyme. The RT product was denaturated at 95°C for 5 min. PCR amplification was carried out in 30 cycles using denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1.5 min using a thermal cycler (Bio-Rad, USA). The final extension step was done at 72°C for 10 min. To fractionate cDNA fragments, 5 µl of PCR reaction mixture was loaded to 1% agarose gel and electrophoresed for 30 min in TAE buffer, containing 0.5 mg/ml ethidium bromide. DNA marker III of 4.5 kb was used as size marker for the determination of the length of the amplified fragments. PCR products were extracted from the gels with E.N.Z.A ®Gel extraction kit (Omega, USA) following the manufacturer's instruction.

The cDNA were then ligated directly into TA cloning vector system pMD19-T simple vector (TaKaRa, Dalian, China) and used to transform competent *Escherichia coli* strain, DH5α, following the manufacturer's instruction. The recombinant colonies were selected by LB agar plates containing 100 µg/ml Amp and the recombinant plasmids were extracted with E.Z.N.A ®Plasmid Miniprep Kit (Omega, USA) and identified by PCR, restriction enzyme digestion and sequencing (TaKaRa).

Multiple alignments and phylogenetic analysis

Comparison of the sequences with published sequences of

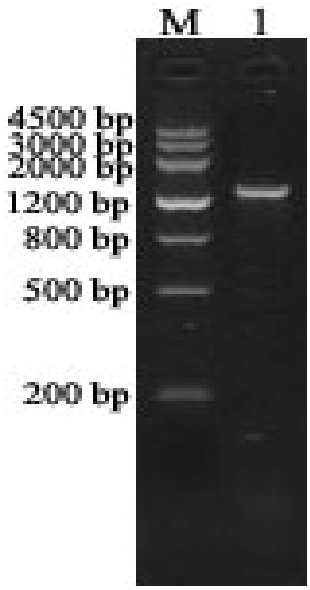

Figure 1. Ethidium bromide stained agarose gel electrophoresis of PCR products of giant panda rotavirus VP6 gene using specific primers. Lane M, DNA marker; lane 1, PCR products of giant panda rotavirus VP6 gene.

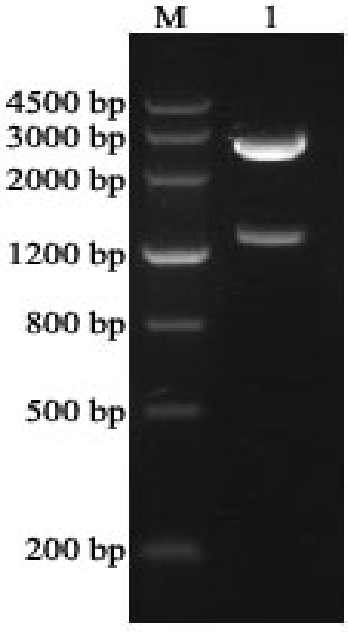

Figure 2. Ethidium bromide stained agarose gel electrophoresis of characterization of the recombinant plasmid pMD19-T-VP6 by restriction digestion. Lane M, DNA marker; lane 1, pMD19-T-VP6 digested with EcoR I and Xho I.

rotavirus members available in the GenBank database was carried out. The rotavirus VP6 gene sequences were used in multiple alignments and phylogenetic analysis for which complete sequences are presently available from the NCBI nucleotide sequence databases. In the case of viral VP6 gene, our analysis was performed with eight rotavirus species available in the GenBank. Multiple sequence alignments and sequence similarity calculations between aligned nucleotide and amino acid sequences were performed using computer software program (DNAstar Inc., Madison, WI, USA). The phylogenetic trees were reconstructed on aligned nucleotide sequences by using the Clustal W program.

RESULTS

Cloning of giant panda rotavirus VP6 gene

Amplification of the VP6 gene of giant panda rotavirus by RT-PCR using primer pairs R1-F1 generated a specific DNA band of 1356 bp (Figure 1), as expected. Then, the PCR products were extracted by E.N.Z.A ®Gel extraction kit (Omega, USA) and cloned into pMD19-T simple vector, thus the recombinant plasmid was constructed and designated as pMD19-T-VP6. The result of the enzyme digestion of the recombinant plasmid is shown in Figure 2. The sequencing results show that the PCR product was 1356 bp in length (Figure 3), and contain an open reading frame (ORF) of 1194 bp in size, encoding a putative polypeptide of 397 amino acids and predicted M_W of 44.2 kDa. The complete nucleotide sequence of giant panda rotavirus VP6 gene has been submitted in the GenBank Database and was assigned an accession number GU188283. So far, a number of other species of RV VP6 gene have been cloned and sequenced, but the giant panda rotavirus VP6 gene sequence has not been reported. This study reports the initial characterization of the giant panda rotavirus VP6 gene from giant panda. In our research, the high fidelity Taq enzyme (TaKaRa Ex Taq^{TM}) was used to decrease the error rate of the PCR.

Sequence analysis

Sequence analysis indicated that the nucleotide sequence of the giant panda rotavirus VP6 gene ORF was 1194 bp in length and had a base pair composition of 386 adenine (32.33%), 208 cytosine (17.42%), 237 guanine (19.85%), 363 thymine (30.40%) and a GC content of 37.27%.

To analyze the phylogenetic relationships of giant panda VP6 gene with different species rotavirus (Table 1), we constructed a phylogenetic tree using the VP6 gene sequences of different species. A representative minimal tree for the VP6 is shown in Figure 4. The eight rotaviruses were separated into two large groups. For further comparison, deduced amino acid sequences were assembled into multiple alignments with the help of MEGALIGN of DNASTAR using clustal W program (Figure 5).

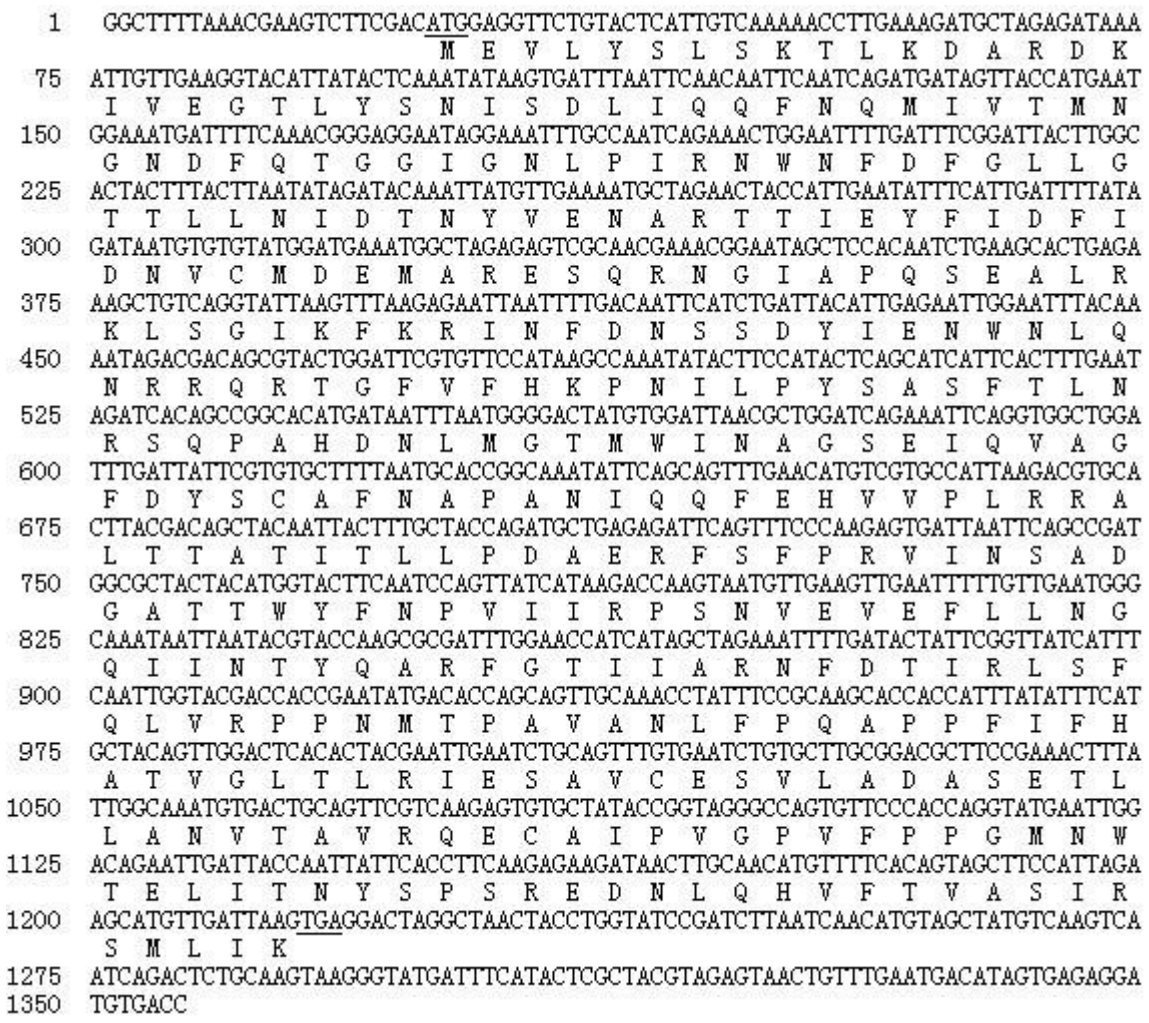

Figure 3. The nucleotide (GenBank accession numbers: GU188283) and deduced amino acid sequences for giant panda rotavirus VP6.

DISCUSSION

Rotavirus is the most common viral agents of acute gastroenteritis in humans and in a large variety of animals worldwide (Estes, 2001; Gentsch et al., 2005; Santos and Hoshino, 2005). Since 1969, Mebus et al. found rotavirus from bovine diarrhea stool; so far, people had isolated rotavirus from bovine, porcine, human, equine, canine, c aprine, cervine, rabbit, feline, murine, avian, simian, turkey, parrot and so on. According to report, the rotavirus positive infection rate of wean piglets was 80% and mortality was 15% in USA. The Asian Rotavirus Surveillance Network reported that overall, 45% of diarrhoea admissions in Asian region were positive for rotavirus in nine countries and regions of Asia (2008), and led to 44,000 young children death every year (Parashar et al., 2003).

According to statistics results, now there are approximately 2500 giant pandas alive in the world, and 1600 of which were hand-fed. As the most precious animal in the word, the health of which has been a topic of concern to human, and also the key topic for researchers. In our research, we cloned and sequenced VP6 gene of giant panda rotavirus, and sequence analysis was carried out by bioinformatics software. The results of nuclear acid sequence comparison of different species showed that the giant panda rotavirus VP6 gene was most closely related to porcine rotavirus (98.7%) and human rotavirus (97.0%). The phylogenetic relationship of rotavirus analysis showed that the giant panda rotavirus VP6 gene is most closely related to porcine and human rotavirus. These data indicate that giant panda rotavirus had higher genetic relationship with porcine rotavirus and human rotavirus than other species rotavirus. That is to say, giant panda rotavirus might have come from porcine and human. Giant panda is closely related to human, thus bringing new infection pathway of rotavirus dissemination.

Table 1. Nucleotide and deduced amino acid sequence identity among different species rotavirus. Sequence identity was determined by the clustal W program in the MegAlign program of Lasergene (DNASTAR). The upper right triangle is the nucleotide sequence identity (shown in bold font) and the lower left triangle is the deduced amino acid sequence identity.

Species accession number	Giant panda GU188283	Porcine FJ617209	Human DQ873675	Bovine GU384194	Canine EU708916	Feline GU827410	Simian AY187029	Avian EF687020
Giant panda	-	**95.1**	**89.2**	**76.3**	**76.0**	**76.0**	**76.7**	**64.1**
Porcine	98.7	-	**90.1**	**76.5**	**76.5**	**77.6**	**77.1**	**64.1**
Human	97.0	98.0	-	**76.5**	**76.7**	**77.3**	**77.7**	**64.5**
Bovine	90.2	90.7	91.0	-	**78.3**	**87.6**	**85.2**	**64.0**
Canine	89.2	89.7	89.9	93.7	-	**77.9**	**78.1**	**62.9**
Feline	90.5	91.0	91.2	98.5	93.5	-	**84.3**	**64.1**
Simian	89.9	90.5	90.7	96.2	92.0	97.0	-	**64.7**
Avian	72.6	73.4	74.6	73.6	72.4	74.4	74.1	-

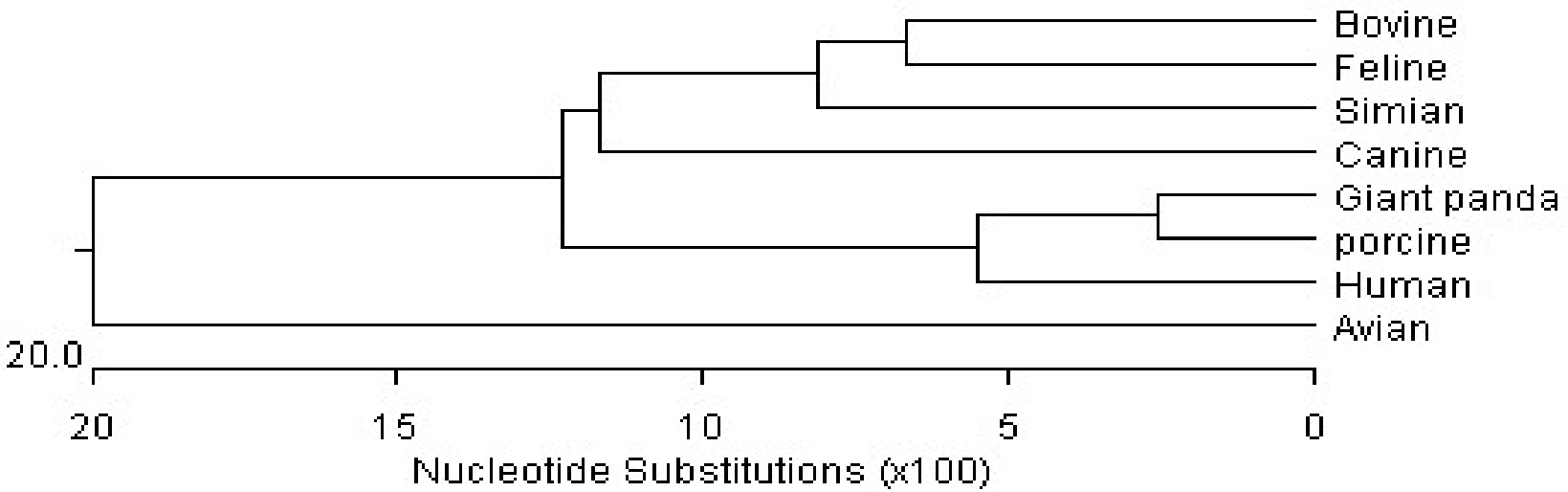

Figure 4. Phylogenetic tree of the nucleotide acid sequences of the VP6 genes of giant panda rotavirus and seven other species rotavirus obtained using the MEGALIGN program in LASERGENE (DNAStar 6.0).

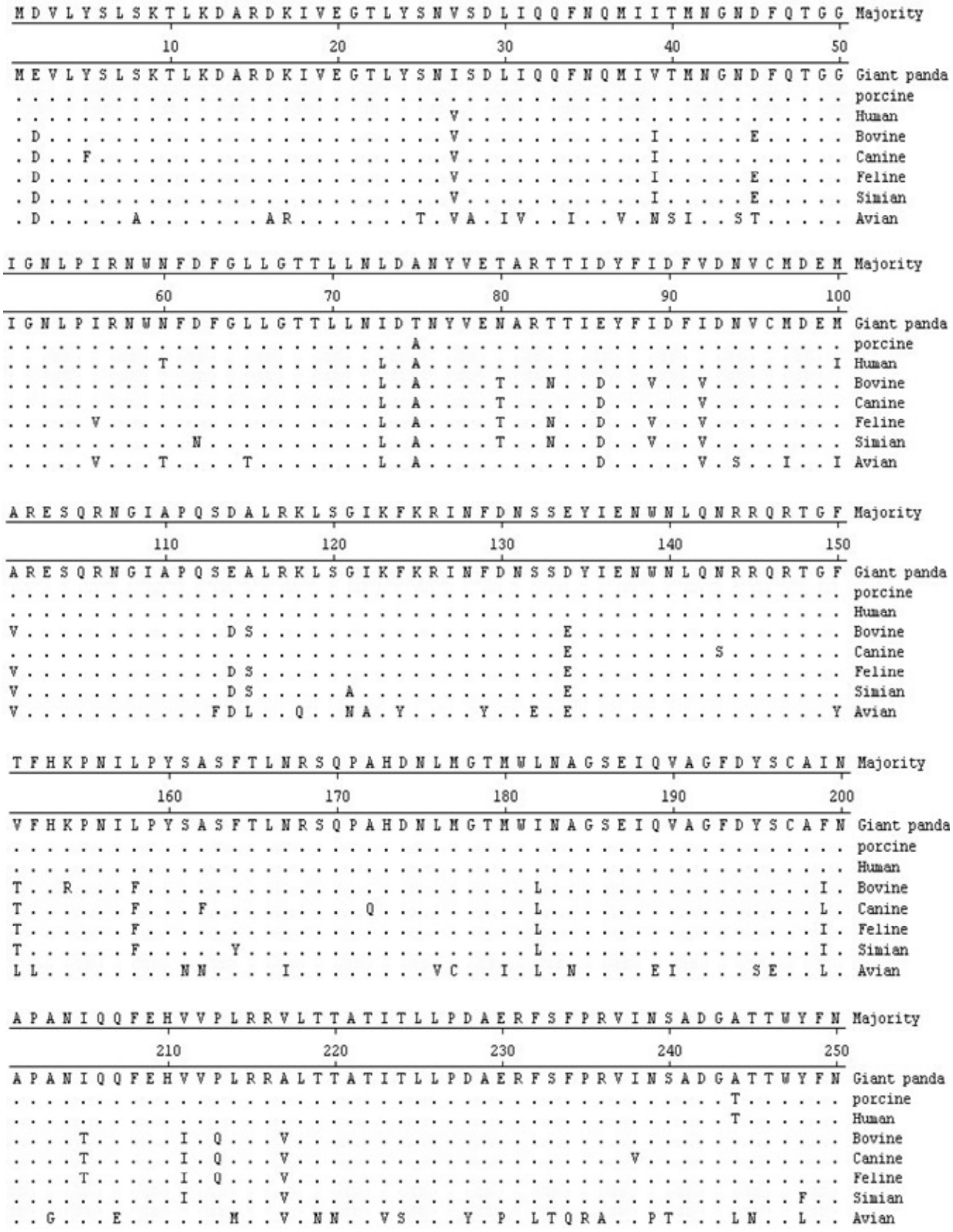

Figure 5. Multiple sequence alignment of deduced amino acid sequences of VP6 from different species of rotavirus including giant panda rotavirus using Clustal W program.

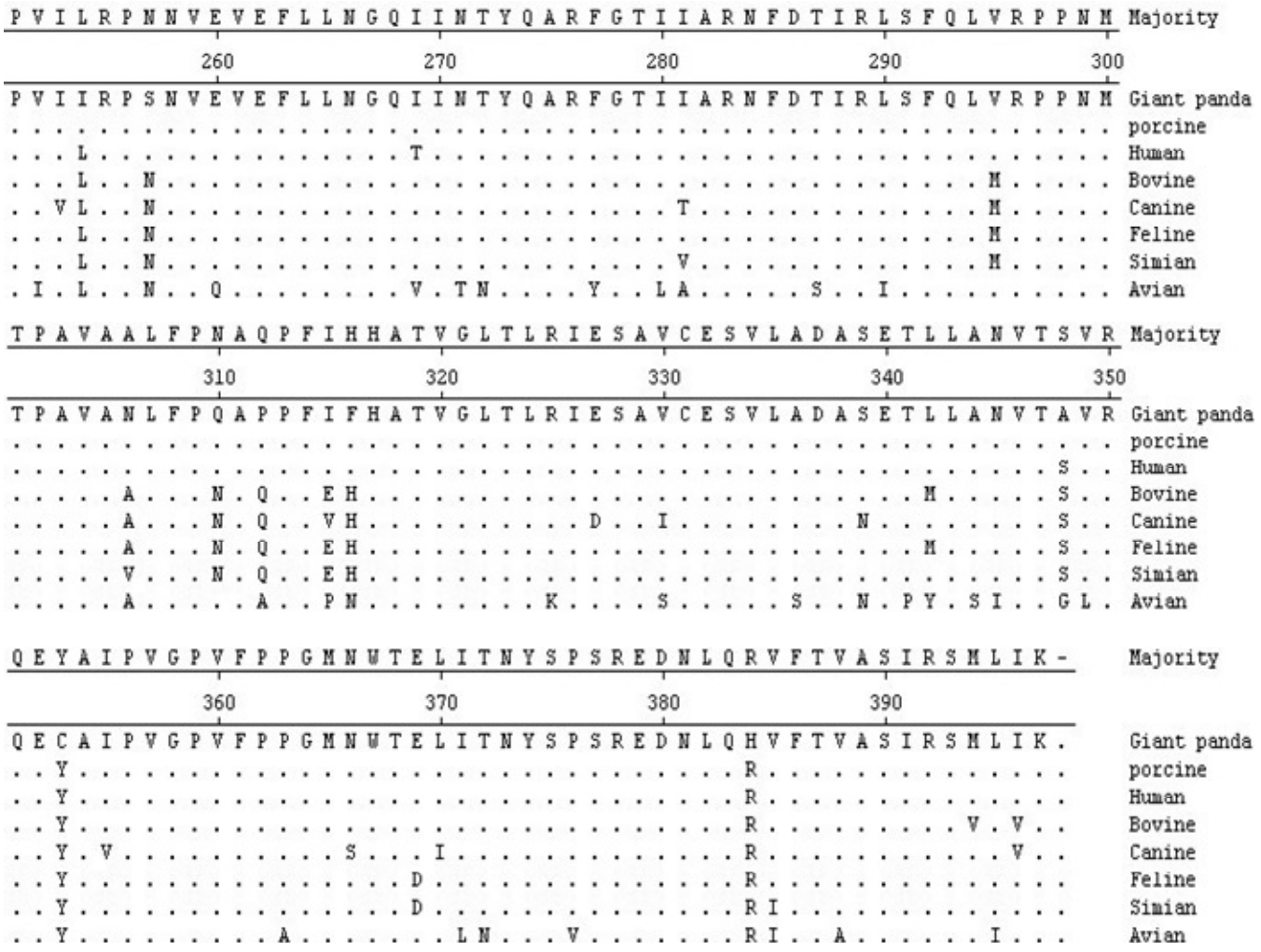

Figure 5. Contd.

Multiple alignment of amino acid sequences showed many substitutions dispersed all along the length of the protein. Among all the rotavirus, giant panda rotavirus showed three unique aa substitutions including A75T, Y353C and R384H; it implies that rotaviruses are genetically closely related.

In conclusion, we reported the initial characterization of the giant panda rotavirus VP6 gene in this study. Sequence analysis indicated that giant panda rotavirus, human rotavirus and porcine rotavirus were mostly related, maybe they have genetic relationship. We are expecting future analyses of more genes and additional virus species to add detail to the study, and provide more useful information for the control of rotavirus dissemination.

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