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Full Length Research Paper

Cloning and phylogenetic analysis of the NSP1~5 genes of giant panda rotavirus strain CH-1 isolated in China

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Rotavirus (RV), a member of the genus *Rotavirus* of the family Reoviridae, is an important causative agent of diarrhoea diseases of human and animals worldwide. 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). But very limited knowledge about animal diarrhoea diseases caused by rotavirus was published until now. Valuation of genetic relations between human and animal rotavirus isolates is very limited. To better understand the rotavirus CH-1 strain isolated from diarrheic faeces of giant panda in 2008, we cloned the non-structural protein (NSP) 1~5 complete coding sequence of the giant panda rotavirus (GPRV), and sequenced NSP1~5 genes (GenBank accession number: NSP1, GU205762; NSP2, GU188281; NSP3, GU329525; NSP4, GU188282; NSP5, GU329526). Based on these information and data from GenBank of other genus of RV NSP1~5 genes, phylogenetic analysis were realized. The phylogenetic tree revealed that GPRV NSP1~5 genes were close to that of porcine rotavirus, bovine rotavirus and human rotavirus. This research may provide some useful information helping us understanding giant panda rotavirus.

Key words: Giant panda rotavirus isolate strain CH-1, NSP1~5 genes, cloning, phylogenetic analysis.

INTRODUCTION

The giant panda (*Ailuropoda melanoleuca*) is one of the world's most recognized and threatened animals on the planets (O'Brien et al., 1994; Jianjuan et al., 2001). Currently, giant pandas are restricted to the isolated Qinling, Minshan, Qionglai, Daxiangling, Xiaoxiangling and Liangshan mountains (Zhang and Wei, 2006), of which the wild population is estimated to be about 1600 and the amount of hand-feeding is about 500.

Among all the diseases, diarrhoea is one of the most serious diseases for giant panda. RV is known to be major agents of severe acute gastroenteritis in infants and young animals. Globally, RV is responsible for enormous morbidity and is estimated to cause 114 million episodes of diarrhoea per year (Grimwood and Buttery, 2010), it remains a major health problem worldwide. Once giant panda infected RV, there was no sign before invasion, but suddenly apastia, intense disgorging, typical

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Table 1. Primers used in PCR amplification.

Gene	Primer sequence	Products length
NSP1	F1: 5'- AAGTCTTGTGGAAGCCA-3' R1: 5'- TAGGCGCTTCTCTAGTG-3'	1532 bp
NSP2	F2: 5'- TAAAGCGTCTCAGTCGCCGT-3' R2: 5'- CGCTTTCTATTCTTGCTTCG-3'	1042 bp
NSP3	F3: 5'-GCTAATGCTTTTCAGTGGTTG-3' R3: 5'-TATTGTGCTCATAGAGGGTC-3'	1014 bp
NSP4	F4: 5'-GGCTTTTAAAAGTTCTGT-3' R4: 5'-GGTCACACTAAGACCATT-3'	750 bp
NSP5	F5: 5'- GGCTTTTAAGCGCTACAGT-3' R5: 5'-GGTCACAAAACGGGAGTG-3'	664 bp

symptom of disease such as water-like diarrhoea, abdominal gaseous distention, diarrhoea protraction and so on appeared, eventually died of multi-organ nonfunction (Wang et al., 2008).

Rotavirus is a triple-layered icosahedral protein capsid surrounding a genome of double-stranded (ds) ribonucleic acid (RNA), of approximately 70 nm in diameter, belonging to the Reoviridae family. The rotavirus genome comprises 11 segments of dsRNA and encodes six structural proteins (VPs) and five non-structural proteins (NSPs) several of which are RNA-binding protein (Pesavento et al., 2009). The NSPs of rotavirus were closely related to the viral replication.

In this research, we clone and sequence the giant panda rotavirus NSP1~5, and compare with other genus of rotavirus, construct phylogenetic tree, find the phylogenetic relationship of giant panda rotavirus with other species rotavirus, in order to find the infect pathway to giant panda, and provide information helping people understanding giant panda rotavirus.

MATERIALS AND METHODS

Strains, plasmids

The *Escherichia coli* DH5α and plasmid pMD19-T simple vector were used for cloning and amplification of NSP1~5 cDNA, were from Invitrogen and TaKaRa, respectively. *LA Taq*™ DNA polymerase were purchased from TaKaRa and used according to supplier's recommendations.

Chemicals

RNAiso PLus, and PrimeScript™RT reagent Kit were from TaKaRa. E.Z.N.A.® Gel Extraction Kit, E.Z.N.A.® Plasmid Mini Kit II were from Omega, Luria-Bertani (LB) broth media was from Life Technologies.

Virus and viral RNA

The giant panda RV strain CH-1 was isolated from Chengdu Research Base of Giant Panda Breeding and propagated in MA-104, a fetal monkey kidney cell line, in the presence of 1 μg/ml trypsin, at 37°C for 2 to 3 days. The cell debris was removed by low speed centrifugation and total virus RNA was purified from culture supernatant according to the instruction of RNAiso PLus. In brief, 600 μl of RNA iso PLus solution was added to 400 μl of the culture supernatant and homogenized, stood at room temperature for 5 min. Then, 120 μl of chloroform was added and the tube was placed in a shaker for 5 min, stood at room temperature for 5 min, centrifuged at 13000 g for 15 min at 4°C, transfer the clean supernatant into a new tube, add adqulis volume isopropyl alcohol, gently mixed by inverting and rotating the tube several times, stood at room temperature for 10 min, centrifuged at 13000 g for 10 min at 4°C, then add 1 ml of 75% alcohol to the tube to wash the precipitation, centrifuged at 13000 g for 5 min at 4°C, discarded clean supernatant, remained the precipitation and dryed, added 40 μl of TE buffer to dissolve it, stored at -20°C.

Reverse transcription reaction, polymerase chain reaction (PCR) and cDNA cloning of NSP1~5

Primers (Table 1) were designed to copy the NSP1~5 gene that codes for non-structure protein of rotavirus.

First-strand cDNA was synthesized from total RNA using the PrimeScript™RT reagent Kit (TaKaRa) according to the manufacturer's instruction. Giant panda rotavirus strain CH-1 NSP1~5 cDNA was amplified using *LA Taq*™ DNA polymerase (TaKaRa) and the pairs of primers F1/R1, F2/R2, F3/R3, F4/R4, F5/R5 (Table 1). NSP1, amplification consisted of 30 cycles of 30 s at 95°C, 30 s at 50°C, 100 s at 72°C; NSP2, amplification consisted of 30 cycles of 30 s at 95°C, 30 s at 48°C, 70 s at 72°C; NSP3, amplification consisted of 30 cycles of 30 s at 95°C, 30 s at 51°C, 70 s at 72°C; NSP4, amplification consisted of 30 cycles of 30 s at 95°C, 30 s at 48°C, 50 s at 72°C; NSP5, amplification consisted of 30 cycles of 30 s at 95°C, 30 s at 50°C, 40 s at 72°C. The amplification products were separated on a 1% agarose gel and visualized by ethidium bromide staining. The positive PCR products were agarose gel purified and ligated into pMD19-T simple vector, the resulting plasmid, was transformed into competent *E. coli* DH5α

cells, positive transformants were selected by ampicillin, and the clones were identified by PCR, and verified by nucleotide sequencing, Each fragment was sequenced from different PCR products at least three times.

Multiple alignments and phylogenetic analysis

Comparison of the sequences with published sequences of members of rotavirus available in the GenBank database was carried out. The rotavirus NSP1~5 gene sequences used in multiple alignments and phylogenetic analysis for which complete sequences are presently available from the NCBI nucleotide sequence databases (Table 2). In this research our analysis was performed with different species rotavirus available in the GenBank. The phylogenetic trees were reconstructed on aligned nucleotide sequences by using the ClustalW method.

RESULTS

Cloning and sequencing results of NSP1~5 gene of giant panda rotavirus strain CH-1

Amplification of the NSP1~5 genes of giant panda rotavirus by RT-PCR using primer pairs R1/F1, F2/R2, F3/R3, F4/R4, F5/R5 generated specific DNA bands of 1532 bp, 1042 bp, 1014 bp, 750 bp, 664 bp (fig. 1), respectively as expected. And then the PCR products were extracted by E.N.Z.A. ®Gel extraction kit (Omega) and cloned into pMD19-T simple vector (TaKaRa), thus the recombinant plasmid was constructed, the positive clones were selected by LB plates contain 100 μg/ml ampricillin, and then sequenced by TaKaRa. The complete nucleotide sequence of giant panda rotavirus NSP1~5 genes has been submitted in the GenBank Database and was assigned accession number GU205762, GU188281, GU329525, GU188282, GU329526, respectively (Figure 1).

Phylogenetic analysis of the NSP1 gene of giant panda rotavirus strain CH-1

Figure 2 shows the phylogenetic relationships of the NSP1 genes of giant panda rotavirus strain CH-1 isolated in China with other genus rotavirus NSP1 sequences available from GenBank. In this research, we analyzed the phylogenetic relationships of giant panda, porcine, human, bovine, equine, canine, feline, simian, rhesus, avian, and found that giant panda rotavirus was more closely related to porcine, bovine and human rotavirus (Figure 2).

Phylogenetic analysis of the NSP2 gene of giant panda rotavirus strain CH-1

Figure 3 shows the phylogenetic relationships of the NSP2 gene of giant panda rotavirus strain CH-1 isolated in China with other genus rotavirus NSP2 sequences available from GenBank. In this research, we analysis the phylogenetic relationships of giant panda, porcine, human, bovine, canine, feline, simian, rhesus, avian, and found that giant panda rotavirus was more closely related to porcine, bovine and human rotavirus (Figure 3).

Phylogenetic analysis of the NSP3 gene of giant panda rotavirus strain CH-1

Figure 4 shows the phylogenetic relationships of the NSP3 genes of giant panda rotavirus strain CH-1 isolated in China with other genus rotavirus NSP3 sequences available from GenBank. In this research, we analysis the phylogenetic relationships of giant panda, porcine, human, bovine, canine, feline, simian, avian, and found that giant panda rotavirus was more closely related to porcine, bovine and human rotavirus (Figure 4).

Phylogenetic analysis of the NSP4 gene of giant panda rotavirus strain CH-1

Figure 5 shows the phylogenetic relationships of the NSP4 genes of giant panda rotavirus strain CH-1 isolated in China with other genus rotavirus NSP4 sequences available from GenBank. In this research, we analysis the phylogenetic relationships of giant panda, porcine, human, bovine, canine, feline, simian, avian, and found that giant panda rotavirus was more closely related to porcine, bovine and human rotavirus (Figure 5).

Phylogenetic analysis of the NSP5 gene of giant panda rotavirus strain CH-1

Figure 6 shows the phylogenetic relationships of the NSP5 genes of giant panda rotavirus strain CH-1 isolated in China with other genus rotavirus NSP5 sequences available from GenBank. In this research, we analysis the phylogenetic relationships of giant panda, porcine, human, bovine, canine, feline, simian, avian, and found that giant panda rotavirus was more closely related to porcine, bovine and human rotavirus (Figure 6).

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). So far, people had isolated rotavirus from bovine, porcine, human, equine, canine, caprine, rabbit, avian, simian, turkey, parrot and so on. Globally, rotavirus is responsible for enormous morbidity and is estimated to cause 114 million episodes of diarrhoea per year (Grimwood and Buttery, 2007), lead to 440,000 young children die of rotavirus infection. And it is reported that, 80% of piglets were positive for rotavirus,

Table 2. The gene sequences used for analysis.

Gene	Virus strain	Isolate	GenBank no
NSP1	CH-1	Giant panda	GU205762
	DC1497	Human	FJ947344
	OSU	Porcine	D38153
	KJ338-1	Bovine	FJ206221
	CU-1	Canine	EU708918
	BA222	Feline	GU827412
	PTRV	Simian	FJ422135
	RRV	Rhesus	RRU08433
	H1	Equine	ERU23728
	PO-13	Avian	AB009633
NSP2	CH-1	Giant panda	GU188281
	DC133	Human	FJ947270
	SB1A	Porcine	EU169872
	KJ58-1	Bovine	FJ206134
	CU-1	Canine	EU708919
	BA222	Feline	GU827413
	PTRV	Simian	FJ422139
	RRV	Rhesus	GU933624
	PO-13	Avian	AB009625
NSP3	CH-1	Giant panda	GU329525
	SE584	Human	EF672607
	OSU	Porcine	X81431
	KJ9-1	Bovine	FJ206226
	CU-1	Canine	EU708920
	BA222	Feline	GU827414
	PTRV	Simian	FJ422137
	PO-13	Avian	AB009626
NSP4	CH-1	Giant panda	GU188282
	RMC/G7	Human	AY601542
	OSU	Porcine	D88831
	KJ338-1	Bovine	FJ206163
	CU-1	Canine	EU708921
	BA222	Feline	GU827415
	H1	Equine	AF144800
	PTRV	Simian	FJ422140
	AvRV-1	Avian	AY062937
NSP5	CH-1	Giant panda	GU329526
	G11S-S79	Human	EF590985
	CMP034	Porcine	DQ916134
	KJ330-1	Bovine	FJ206099
	CU-1	Canine	EU708922
	BA222	Feline	GU827416
	PTRV	Simian	FJ422141
	PO-13	Avian	AB009627

and 15% deaths due to rotavirus in USA. When giant panda infect rotavirus, there were no signs

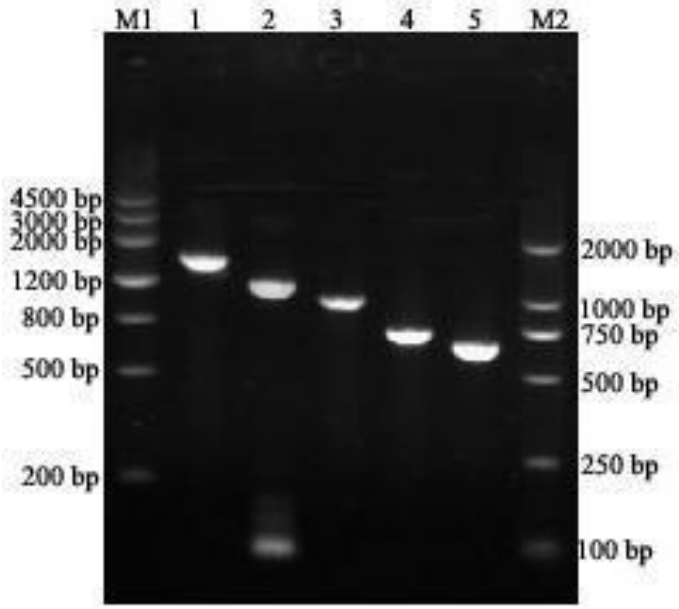

Figure 1. RT-PCR products of giant panda rotavirus NSP1~5 genes. Line M1, DNA marker of 4500 bp; lane 1, amplification product of NSP1; lane 2, amplification product of NSP2; lane 3, amplification product of NSP3; lane 4, amplification product of NSP4; lane 5, amplification product of NSP5; lane M2, DNA marker of 2000 bp.

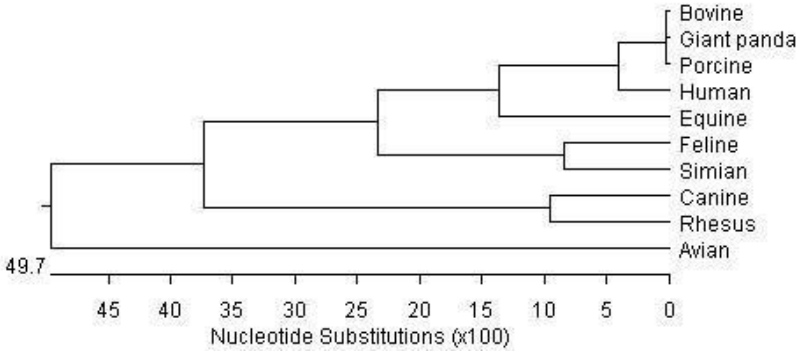

Figure 2. Phylogenetic relationships of the NSP1 genes from different genus.

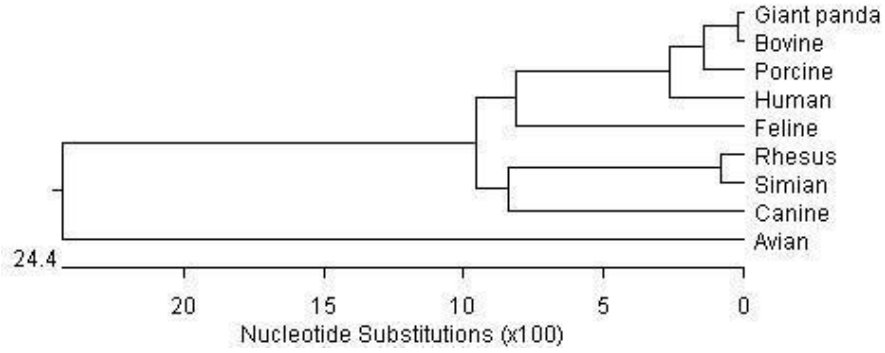

Figure 3. Phylogenetic relationships of the NSP2 genes from different species.

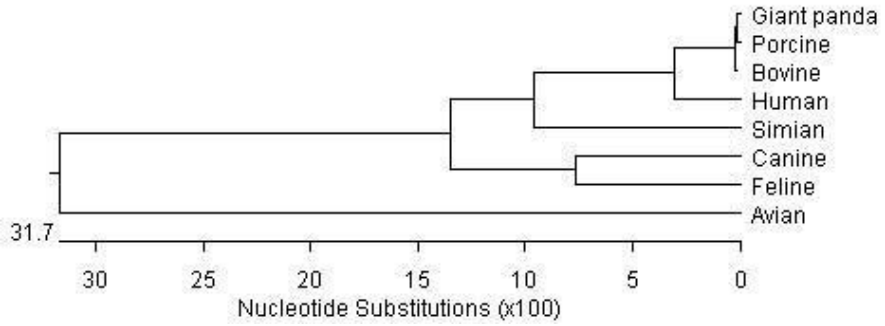

Figure 4. Phylogenetic relationships of the NSP3 genes from different species.

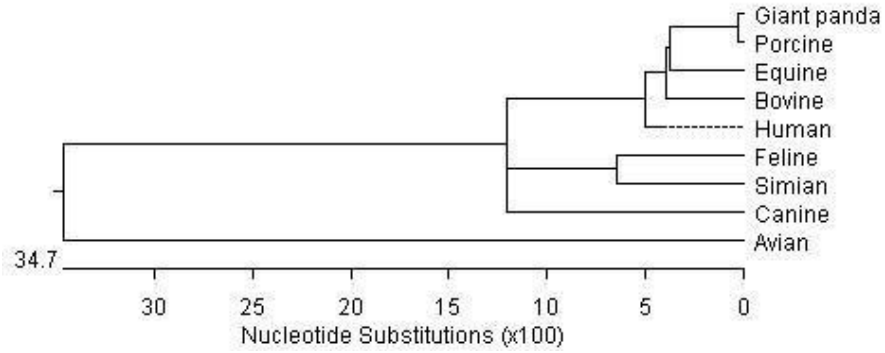

Figure 5. Phylogenetic relationships of the NSP4 genes from different species.

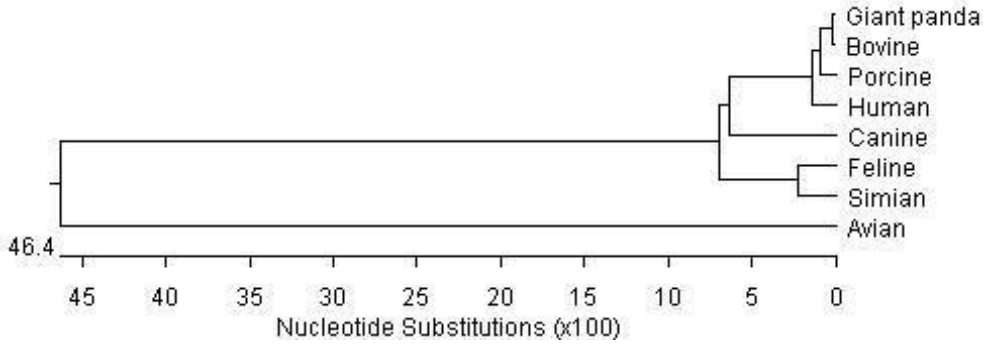

Figure 6. Phylogenetic relationships of the NSP5 genes from different species.

before invasion, but suddenly appear apastia, intense disgorging, then appear key feature of disease such as water-like diarrhoea, abdominal gaseous distention, diarrhoea protraction and so on, eventually die of multi-organ nonfunction. This bring huge dangerous to giant panda. The aim of our study was to analyze the NSP1~5 genes of giant panda rotavirus, in order to investigate the evolution of those genes.

Rotaviruses are the major cause of severe gastroenteritis in human and animals. The rotavirus genome is composed of eleven segments of double-stranded RNA and can undergo genetic reassortment during mixed infections, leading to progeny viruses with novel or atypical phenotypes. There are numerous descriptions of rotavirus strains isolated from human and animals that share genetic and antigenic features of viruses from heterologous species. In many cases, genetic analysis by hybridization has clearly demonstrated the genetic relatedness of gene segments to those from viruses isolated from different species

(Palombo, 2002). The results above indicate that the NSP1~5 genes of giant panda rotavirus were all closely related to porcine, human, and bovine rotavirus. This suggested that the giant panda rotavirus strain CH-1 maybe one genetic reassortment virus, and maybe mixed infection from human, bovine, and porcine. As ornamental animals, giant panda is closely contact with human, so, we thought that giant panda rotavirus may come from human, but the mechanism of transmission need further study and research.

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