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Original Article

Antibody Production of Baculovirus-expressed VP6 from Porcine Group C Rotavirus

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The emerging pathogen, group C rotavirus (RVC) has been reported to cause acute diarrhea. But there was the limitation on the detection and monitoring for the absence of rapid sensitive diagnosis system. For the molecular biology study and diagnostic system development, we could detect porcine RVC by reverse transcriptase PCR (RT-PCR) analyses from 60 diarrheal disease porcine stool samples. VP6 full length RT-PCR product (CA-2 RVC, 1352 bp) was cloned and compared the nucleotide and deduced amino acid sequences with those of previously reported other porcine, human, and bovine rotavirus group A, B and C strains. Analyses data showed >82% homology on the nucleotide sequences and >90% homology on the deduced amino acid sequences with other RVCs. Recombinant baculovirus was prepared with cloned PCR product corresponding to VP6 coding sequence (CDS) (position 22~1206) into BaculoDirect™ C-term linear DNA, and used for the transfection of insect cells. The polyclonal antibody was produced from mice with purified recombinant VP6 and confirmed with western blot. Both of VP6 antigen and antibody, are useful for the development of rapid diagnostic system against RVC.

Key Words: Porcine group C rotavirus, Baculovirus, VP6, Polyclonal antibody

INTRODUCTION

Rotavirus has been known as the major causative agent of acute dehydrating diarrhea in humans as well as various domestic animals (1). Rotaviruses are the member of *Reoviridae* family which has non encapsulated triple-layered particle containing double stranded linear RNA (dsRNA) genome. The external capsid consists of VP4, a protease sensitive hemagglutinin and VP7, a glycoprotein functioning as a neutralizing antigen. The inner capsid is composed of antigenicity providing protein, VP6 (2, 3). Rotaviruses can be antigenically classified into seven distinct groups, A-G by common group antigen VP6. Group A rotaviruses have been known as the leading cause of childhood diarrhea, associated with 350,000~710,000 deaths/year mainly in the developing countries (4). Group B rotaviruses detected first in China infect mostly adults and older children causing diarrhea (5, 6).

Group C rotavirus (RVC) has been reported to cause mild disease with fewer hospitalizations in children compared to

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group A rotavirus (7). RVC was reported first from pigs intestinal contents samples in 1980 and have been detected in humans, ferrets, and cattle (8~12). While several survey studies reported lower detection rates of RVC in fecal specimens of children with diarrhea compared to group A rotavirus, there is need to consider about unavailability of adequate diagnostic system (13~19). The most frequently used standard system for virus detection, PCR or nested PCR sometimes showed insensitiveness problem on the diagnosis of RVC for the unstable viral capsid proteins and the empty viral particles (19~21). And also some current ELISA assay has problem of specificity and sensitivity for the inability to recognize RVC VP6. So it is necessary to develop a more convenient and economical tool, like a RVC specific enzyme immunoassay (EIA), or rapid one-step immunochromatographic assay with higher accuracy to monitor and block the propagation of RVC. For the rotavirus intermediate capsid layer forming VP6 is the most abundant protein and group antigen used for the classification of rotavirus group, VP6 region was selected and used for antibody production (22). In this study, the antibody was produced using cloned porcine RVC full length VP6 gene as the basic work for the development of sensitive detection system.

MATERIALS AND METHODS

RNA extraction from porcine fecal specimens and RT-PCR

For the selection of RVC positive samples, RNAs were extracted with QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) from 60 stool samples of diarrheic pigs. The RT-PCR using Qiagen One Step RT-PCR kit was performed with the primer set designed for the amplification of 340 bp region of porcine RVC VP6 gene (F: 5'-^{1014}GAATCA-CATAGAACGGACT^{1032}-3', R: 5'-^{1334}GAAATGTGAAC-TATGTGGCT^{1353}-3') (23). After the reverse transcription with 15 min at 95℃ followed by 30 min at 50℃, 35 amplification cycles were performed with 50 sec at 94℃, 1 min at 50℃, and 1 min at 72℃, followed by 10 min at 72℃ for the final extension using Thermo Hybaid Px2 (Thermo Hybaid, Middlesex, UK), and results were analyzed on the 2% agarose gel electrophoresis.

Cloning of full length VP6 gene and molecular analysis

RT-PCR was carried out using Qiagen One Step RT-PCR kit with primer set covering the full length of VP6 gene (F: 5'-^{1}GCATTTAAAATCTCATTCACA^{21}-3', R: 5'-^{1352}AGCC-ACATAGTTCACATTTC^{1337}-3') (24). RT-PCR containing reverse transcription (15 min at 95℃, 30 min at 50℃), amplification (35 cycles of 50 sec at 94℃, 1 min at 52℃, and 1 min at 72℃) and final extension (10 min at 72℃) was performed. Amplified full length VP6 gene was extracted from an agarose gel using Gel/PCR DNA Extraction kit (RBC, Taipei, Taiwan) and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). Plasmid containing full length VP6 gene was used for the transformation of *E. coli* HIT competent cell (RBC). Selected colony on the agar media containing ampicillin was cultured and used for the preparation of plasmid DNA.

Recombinant baculovirus construction

To express recombinant VP6 protein from insect cell using BaculoDirect™ Baculovirus Expression System (Invitrogen, Carlsbad, CA, USA), VP6 gene was amplified with PCR using the primer set designed for coding sequence (CDS) (F: 5'-^{22}ATGGACGTGTTGTTTTCAATT^{42}-3', R: 5'-^{1206}CATCACCATTCTCTTCACGGA^{1186}-3'). The product from the 25 amplification cycles composed of 50 sec at 94℃, 1 min at 50℃, and 1 min at 72℃, after the reaction of 5 min at 95℃ was cloned into pCR8/GW/TOPO® entry vector (Invitrogen). And VP6 cloned entry vector was recombined with BaculoDirect™ C-Term linear DNA containing V5 epitope and 6× His tag for the detection and purification of recombinant protein using LR Clonase™ II Enzyme Mix (Invitrogen). High Five™ insect cells (Invitrogen, 8 × 10^5/well) was seeded in SF-900 II serum-free medium (Gibco BRL, Grand Island, NY, USA) on 6 well plate (Falcon, BD Biosciences, Franklin Lakes, NJ, USA) and cultured to 95% confluence at 27℃ in 5% CO_2 incubator. And then cells were transfected with nucleotide

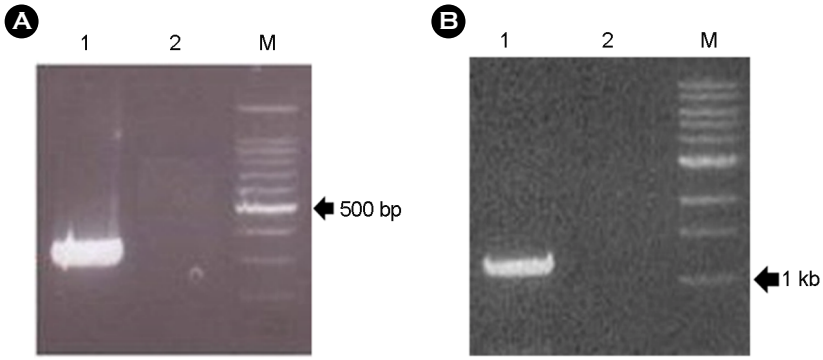

Figure 1. RT-PCR result for the detection of RVC with the 340 bp region of VP6 gene (A) and for the cloning of full length VP6 gene (B). (A) Lane 1, porcine RVC positive (340 bp); 2, negative control; M, 100 bp ladder. (B) Lane 1, full length VP6 (1352 bp); 2, negative control; M, 1 kb ladder.

sequence confirmed recombinant BaculoDirect™ virus DNA using Cellfectin® Reagent (Invitrogen). The expression pattern of recombinant VP6 protein from cell lysate was monitored by 12% SDS-PAGE stained with Coomassie-blue R-250 (Bio-Rad Laboratories, Richmond, CA, USA) and western blot with anti-His antibody (1st antibody; 1:5,000, AbFrontier, Seoul, Korea) and goat anti-mouse IgG HRP (2nd antibody; 1:5,000, Pierce, Rockford, IL, USA) 96 hr after the transfection. Collected viral stock was used for the amplification and scale-up of virus with serial infection.

Recombinant VP6 protein expression and antibody production

To optimize expression and purification condition, 1×10^6 High Five™ cells were infected with amplified viral stock in T75 flask and incubated for various period after infection and two kinds of buffer system [A: 20 mM Tris, 10 mM NaCl (pH 8.0), B: 50 mM NaH_2PO_4, 300 mM NaCl (pH 8.0)] were tested for Ni-NTA Purification System (Invitrogen).

Recombinant VP6 protein from 1 liter scale-up purification procedure was confirmed with 12% SDS-PAGE and western blot and used for polyclonal antibody production. Female Balb/c mice (Charles River Laboratories Japan Inc., Yokohama, Japan) were immunized with intraperitoneal injections three times with 20 μg of recombinant VP6 mixed with complete Freund's adjuvant (Sigma, St. Louis, MO, USA), incomplete Freund's adjuvant (Sigma) and PBS, with 2 week intervals. The activity of mouse polyclonal antibody was confirmed by western blotting.

RESULTS

RVC detection from diarrheic pigs in South Korea and its molecular analysis

One stool sample showed 340 bp on the RT-PCR and was determined to be RVC positive from 60 diarrheic pigs stool samples (Fig. 1A). Full length VP6 of 1352 bp (CA-2 RVC) was obtained from RT-PCR using the positive sample as the template (Fig. 1B), sequenced (Cosmo Co. Ltd, Seoul, Korea) and submitted to GenBank (accession number GQ925781).

Alignment using DNASTAR, EditSeq program showed >83% on the nucleotide sequence and >90% on the deduced amino acid homologies with other previous reported human, porcine and bovine rotavirus group A, B and C strains (24~31). And porcine Cowden strain showed highest score of homology of nucleotide sequence (86.2%) as well as deduced amino acid (93.7%). The homologies with rotavirus group A and B virus strains are much lower than those of RVC strains (Table 1).

Recombinant baculovirus construction and antibody production

The expression of VP6 protein from transfected insect cells with recombinant baculovirus was monitored with 12% SDS-PAGE and western blot. After the confirmation of recombinant VP6 protein, viral stock was collected and used for amplification with serial infections to High Five™ insect cells. The productivity of the expression and purification with various conditions was tested with amplified

Table 1. Comparison of the nucleotide and deduced amino acid sequence homologies of the VP6 gene of CA-2 RVC with other rotavirus strains

Host species	Group	Strain	Accession numbers		Percent homology (%)	
			Nucleotide	Amino acid	Nucleotide	Amino acid
Human	A	B1711	EF554086	ABU49770	51.6	40.4
Human	A	Hun5	EF554108	ABU49772	53.3	41.9
Bovine	A	WC3	AF411322	AAM73767	52.8	41.9
Avian	A	CH2	EF687020	ABV46497	50.2	42.2
Porcine	A	RU172	DQ204741	ABB17170	49.0	40.9
Human	B	WH-1	AY539858	AAT09116	37.6	15.7
Human	B	BANG373	AY238389	AAQ18653	37.6	15.9
Bovine	B	DB176	GQ358713	ADC53099	39.7	16.9
Human	C	Belem	M94155	AAA47339	82.6	90.7
Human	C	Bristol	X59843	CAA42504	82.8	90.7
Bovine	C	Shintoku	M88768	AAA03235	82.4	90.4
Porcine	C	Cowden	M94157	AAA47097	86.2	93.7

Figure 2. Analysis of expression of recombinant VP6 protein from insect cells (A, B) and confirmation of activity of polyclonal antibody (C). (A) 12% SDS-PAGE analysis of recombinant VP6 protein (M, Protein size marker; Lane 1, 2 μg of cell lysate); (B) Western blot analysis of recombinant VP6 protein with anti-HisG antibody (1:5,000) (Lane 1, total cell lysate; 2, supernatant of cell lysate; 3, flowthrough; 4~5, washed; 6~8, eluted); (C) Western blot analysis using mouse polyclonal antibody (1:2,000) (Lane 1, 1.5 μg; 2, 3 μg of recombinant VP6 protein)

viral stock. The most efficient condition for recombinant VP6 protein expression was cells harvested two day after infection and purification with buffer B system (data not shown).

The quality and quantity of 1 liter scale-up recombinant VP6 protein (1.2 mg/ml) was clarified on the 12% SDS-PAGE and western blot (Fig. 2A and B). Western blot with mouse polyclonal antibody produced with recombinant VP6 protein clearly verified the reactivity of antibody (Fig. 2C).

DISCUSSION

In three rotavirus serogroups (A, B, and C) associated with disease in humans, group A rotavirus was known to be the most common cause of acute severe diarrhea in young children. And group B rotavirus also was reported to be involved in diarrhea outbreaks or sporadic cases among children and adults in several countries including China, India, and Bangladesh and is also a common pathogen in animals (32).

RVC human infection has been reported on the sporadic and large outbreaks cases of gastroenteritis of all age groups and distributed globally (33). Even though the RVC was detected first in 1980 from pigs (8), porcine RVC was reported in only few countries (12). The alignment of the nucleotide sequence and deduced amino acid showed the highest homologies of this porcine RVC (CA-2 RVC) with the previous reported representative porcine RVC strains,

Cowden (accession number M94157), compared to other species RVC. But the percent homologies of nucleotide (86.2%) and deduced amino acid (93.7%) also indicate the considerable variation as well as genetic diversity between them.

The emerging human pathogen, RVC has been monitored and reported from several developing countries including Hungary, Turkey, Argentina, Brazil, and Thailand as well as advanced countries like United States and Japan (3, 17, 21, 32, 34~36). But in Korea there was no report about RVC infection in animal or human except only a recent paper about porcine RVC (12).

Detection of RVC using polyacrylamide gel electrophoresis (PAGE) analysis of the double strand RNA needs at least 10^8 to 10^{10} viral particles/ml amounts for the positive result (37, 38). Reverse transcription polymerase chain reaction (RT-PCR) with group C-specific primers is a sensitive and convenient method, however it needs pretreatment of virus samples with experienced technique (7, 17, 34). Diagnosis of RVC has difficulties on the ELISA assay for the absence of antibody against group C-specific antigen VP6 (12).

Rotavirus capsid protein VP6 is the main structural protein consisting of about 50% of virion mass and its significant role in immunological protection has been known (22, 39). And some studies have been reported about functional mapping of protective epitopes in VP6 protein and reveal that the carboxyl terminal region (197 to 397 amino acid) has protective efficiency and the VP6 region of CA-2 RVC was selected (22, 40). BaculoDirect™ Baculovirus Expression System was adapted for it has several advantages like, eukaryotic post-translational modification, proper protein folding and function, high expression levels, easy scale up with high-density suspension culture and it can overcome limitation from protein expression using the bacterial system (41).

For the provision and prevention of spread of hazard RVC including interspecies transmission, it is needed to prepare exact diagnostic system for the public health (42). So, this research is meaningful not only for the detection of porcine RVC in Korea but also for the production of the recombinant antigen from the insect cell line and antibody for the development of diagnostic system. And the further study is needed to apply this antibody to produce effective and confirmed rapid diagnosis kit for the practical application of stool samples.

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