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Original Article

Protein Expression of the Human Norovirus Capsid Gene using the Baculovirus Expression System

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Human norovirus (HuNoV) is the major etiological agent of nonbacterial gastroenteritis worldwide. However, due to the absence of a rapid and sensitive diagnostic system, detection and monitoring have been limited. The HuNoV genome is composed of three open reading frames (ORFs). And major capsid protein, ORF2, is designated as a viral protein 1 (VP1). In this study, the baculovirus expression system was used for expression of the HuNoV capsid protein, VP1. Recombinant baculoviruses can be used as potent tools in HuNoV studies.

Key Words: Human norovirus, Baculovirus expression system

INTRODUCTION

Human norovirus (HuNoV) is in the family *Caliciviridae* and is a non-enveloped positive sense single-stranded RNA with a genome of approximately 7.5 kb. The genome has a small-round structured morphology. HuNoVs are the major etiological agent of nonbacterial gastroenteritis worldwide. Symptoms of HuNoV illness include nausea, vomiting, diarrhea, and some stomach cramping. The illness is usually mild and self-limiting, but the virus is highly infectious and is often transmitted. However, due to the absence of a rapid and sensitive diagnostic system, detection and monitoring have been limited. And HuNoVs could not be cultivated in cell culture nor passed in animal models (1).

The HuNoV genome is composed of three open reading frames (ORFs): ORF1 (nucleotides 5-5104), ORF2 (nucleotide 5085-6731), and ORF3 (nucleotide 6731-7495). ORF1 encodes a nonstructural polyprotein, ORF2, the major capsid protein, and ORF3, the minor capsid protein. Major capsid protein ORF2 is designated as viral protein 1 (VP1), which folds into two major domains: a shell domain (S domain), which is essential for initiation of viral capsid assembly and a protruding domain (P domain), which is important for stabilization of the capsid structure (2). The P domain is further divided into the P1 and P2 sub-domains: P2 has the highest degree of sequence divergence in the genome (3).

NoV can be divided genetically into five genogroups (GI, GII, GIII, GIV, and GV) based on genome sequence; however, GI and GII are the common genogroups in infection of humans. Recently, HuNoV strains belonging to GI and GII were subdivided into at least 15 and 18 genotypes, respectively (4, 5). Of these, HuNoV GII/4 has been reported as the most prevalent genotype in causing acute gastroenteritis in both developed countries and developing

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countries (4).

Effective control of HuNoV infection starts from rapid identification of pathogens. Reverse transcription-PCR (RT-PCR) assays are now used as the common method of HuNoV detection worldwide; however, these methods are time consuming, expensive, and require the use of skillful techniques (4, 6). And, despite many previous studies, diagnosis of HuNoV using the ELISA assay has been difficult due to the absence of relevant antibodies. Therefore, for the sake of public health, we need a quick and reliable diagnostic system. In this study, we selected the three available epitope regions belonging to ORF2. Proteins were expressed by a baculovirus protein expression system that can be used as a system for rapid detection of HuNoVs. The final aim is to construct a rapid diagnostic kit for detection of HuNoV.

MATERIALS AND METHODS

RNA extraction from stool samples and gene amplification by RT-PCR

A stool suspension of approximately 10% was prepared in Dulbecco's Phosphate Buffered Saline (DPBS) using the selected human norovirus positive sample (HuNoV GII/4 positive sample) and precleared by centrifugation at 13,000 rpm for 15 min at 4℃ (7, 8). Genomic RNA was extracted from stool supernatant using the QIAamp Viral RNA mini kit (Qiagen), according to the manufacturer's protocol. RT-PCR was performed for amplification of sequences of the capsid gene of HuNoV using a OneStep RT-PCR Kit (Qiagen) (9). RT-PCR was carried out using the following primer set: epORF2-F (5'-ATGAGCCCCAGCCAGGTCACT-3'), epORF2-R (5'-TAATGCACGCCTGCGCCCCGT-3'), SP1-F (5'-ATGCCCTACCTATCCCATTTG-3'), SP1-R (5'-GCATCTGCCATTTTGTGGTT-3'), P2-F (5'-ATGCTGTCTCCTGTCAACATC-3'), P2-R (5'-GAGCACCCATTGTTGGGGTTC-3'). The amplification conditions were as follows: reverse transcription at 50℃ for 30 min, initial PCR activation at 95℃ for 15 min, 35 amplification cycles with denaturation at 94℃ for 40 sec, annealing at 55℃ for 40 sec, extension at 72℃ for 1 min and final extension at 72℃ for 10 min.

Generation of recombinant baculovirus DNA

Amplicons were cloned into a pCR®8/GW/TOPO® entry vector (Invitrogen). And a clonal pCR®8/GW/TOPO® entry vector was recombined with BaculoDirect™ C-Term linear DNA (Invitrogen) using the BaculoDirect™ System, according to the instructions of the protocol (10).

Cell culture

Insect cells of the *Spodoptera frugiperda* cell line SF-21 were grown in Grace' insect Medium (Gibco) containing 10% heat inactivated fatal bovine serum (FBS) (v/v) (Well gene) and 1% gentamycin (w/v) at 27℃ in an incubator.

Transfection of insect cells

Cells were seeded in a 6-well tissue culture plate at 8×10^5 cells/well with 2 ml of Grace's insect Medium, Unsupplemented (Gibco) without antibiotics and serum and then incubated overnight at 27℃ to allow the cells to fully attach to the bottom of the plate. On the following day, recombinant baculovirus DNA were transfected into SF-21 cells using cellfection®II Reagent (Invitrogen). The cells were incubated at 27℃ for 72 hours until they exhibited cytopathic effects (CPE). Then, 2 ml of medium was collected from each well and centrifuged in order to remove cells and large debris.

Expression and analysis of recombinant protein

SF-21 cells were seeded in 6-well tissue culture plates at 2×10^6 cells/well with 2 ml of complete growth medium (Gibco) and incubated at 27℃ overnight to allow the cells to fully attach to the bottom of the plate.

The recombinant viral stock was infected into SF-21 cells at multiplicities of infection (MOI) of 1. The cells were incubated at 27℃ until more than 80% cells exhibited CPE. Cells and media were harvested when almost cells exhibited CPE, and gently spin in order to pellet the cells. Cell lysate was monitored with analysis by SDS-PAGE stained with Coomassie-blue R-25 (Bio-Rad Laboratories).

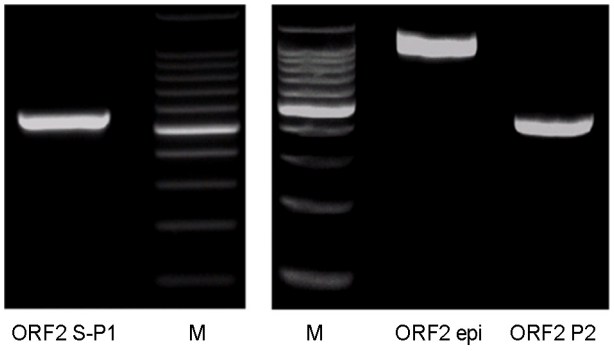

Figure 1. Amplified Fragments of ORF2 S-P1 (543 bp), ORF2 epi (1,224 bp), and ORF2 P2 (347 bp) of the HuNoV capsid gene by RT-PCR (M: DNA size Marker).

RESULTS

Amplification of the target gene

The ORF2 epi region was amplified by RT-PCR (primer set epORF2-F/epORF2-R) and ORF2 S-P1 domain (primer set SP1-F/SP1-R), ORF2 P2 domain (primer set P2-F/P2-R) regions were amplified by PCR. Each of the amplicons was 1,224 bp, 543 bp, and 347 bp size fragments were identified on 1.5% agarose gel electrophoresis (Fig. 1).

Cloning of HuNoV Capsid genes into the entry vector

Capsid protein genes derived from a HuNoV strain and amplified by RT-PCR and PCR were cloned into an *E. coli* entry vector (Fig. 2A). And the positive transformants were screened by restriction enzyme (*Eco*RI) digestion. The expected band size was 1,240 bp for ORF2 epi, 559 bp for ORF2 S-P1, and 403 bp for ORF2 P2, and these were confirmed on 1.5% (w/v) agarose gel electrophoresis (data not shown).

Sequence analysis of the recombinant plasmid

After restriction enzyme digestion, recombinant plasmid DNA sequencing was performed for further confirmation of the inserted fragments. The insert fragments were translated into amino acid sequences in the DNASTAR program and matched with reference strains of NoV GII-4 type (accession number FJ514242) in the MagAlign program.

Generation of recombinant bacmid

The entry clone containing HuNoV capsid genes were LR recombinant reaction with BaculoDirect Linear DNA for generation of recombinant Baculovirus DNA (Fig. 2B). The recombinant Baculovirus DNAs were screened by PCR using the primer set Polyhedrin Forward Primer, V5 Reverse Primer and these were confirmed on 1.5% (w/v) agarose gel electrophoresis (data not shown). The amplicon sizes were 1,474 bp for ORF2 epi, 793 bp for ORF2 S-P1, and 637 bp for ORF2 P2. Recombinant bacmid DNA sequencing was performed for further confirmation of the inserted fragments.

Expression of recombinant protein in SF-21 cells

The positive recombinant bacmid DNAs were transfected into SF-21 cells and analysis of recombinant viral DNA by PCR using specific primers. And expression of HuNoV capsid protein was confirmed by 12% SDS-PAGE gel electrophoresis. The expected band sizes were 54 kDa for ORF2 epi, 29 kDa for ORF2 S-P1, and 23.4 kDa for ORF2 P2 (Fig. 3).

DISCUSSION

HuNoVs are the most common agents of outbreaks of acute gastroenteritis in Korea, and they are transmitted primarily through the fecal-oral route, either by consumption of focally contaminated food or water or by direct person-to-person spread. However, there is no cure for HuNoVs infection and no vaccine (11, 12). Worse still, noroviruses survive freezing, heating to 60℃, and the amounts of chlorine added to public water supplies (13). Nevertheless, HuNoVs are difficult to propagate in cell cultures, and they must be directly detected in clinical specimens and food extracts, which is associated with many problems concerning standardization, including inhibition of enzymes used in RT-PCR (12, 14). However, RT-PCR assays are time consuming, expensive, and require use of skillful techniques (6). And, despite many previous studies, diagnosis of HuNoV using the ELISA assay has been difficult due to

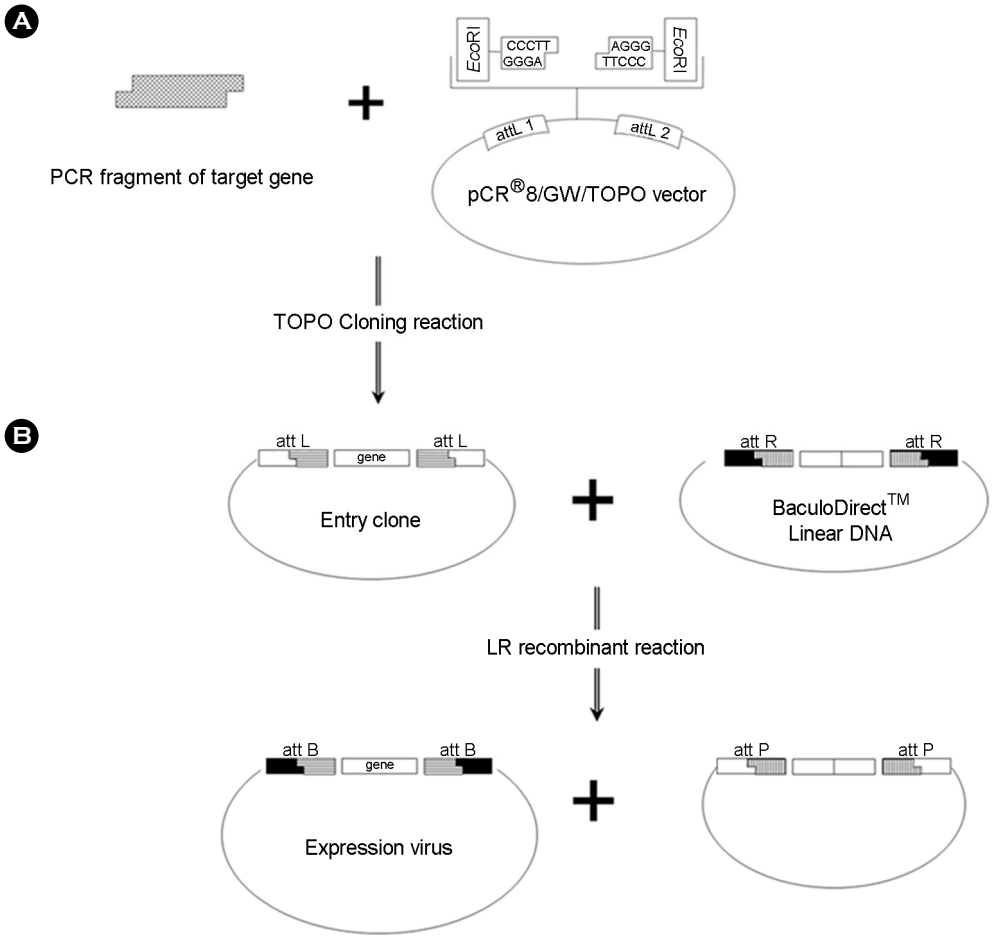

Figure 2. The Gateway cloning strategy. The process comprises of two recombination processes; TOPO cloning reaction (A) and LR recombinant reaction (B).

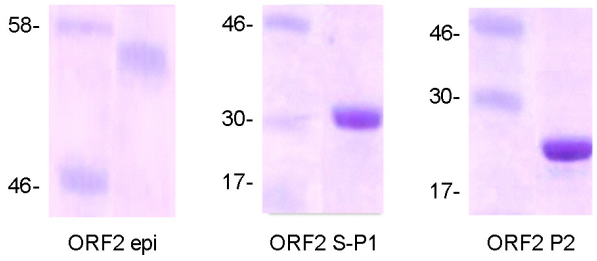

Figure 3. Analysis of expression of recombinant protein from insect cells by SDS-PAGE; ORF2 epi (54 kDa), ORF2 S-P1 (29 kDa), and ORF2 P2 (23.4 kDa).

the absence of relevant antibodies. So, we have to search available epitope region of HuNoV to express relevant antibodies for develop quick and reliable diagnostic system.

In this study, we selected three available epitope regions belonging to ORF2 (the major capsid protein); ORF2 epi (5484-6701), S-P1 (5343-5882), and ORF2 P2 (5916-6296). And proteins were expressed by a baculovirus expression system. The baculovirus expression system is one of the most attractive viral vectors. The baculovirus expression system has been adapted for it has several advantages like, such as 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 (15).

These recombinant proteins can be use development of rapid diagnostic kit for detection of HuNoV. Further study is needed for specific antibody synthesis and application of this antibody for confirmation with a small fragment of recombinant proteins and stool samples. Therefore, for the

sake of public health, a quick and reliable diagnostic system is needed.

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