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Baculovirus expression of HCoV-OC43 nucleocapsid protein and development of a Western blot assay for detection of human antibodies against HCoV-OC43

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Abstract

The nucleocapsid (N) gene of human coronavirus strain OC43 (HCoV-OC43) was amplified by reverse transcriptase-polymerase chain reaction, and cloned in pENTR™/D-TOPO® plasmid. This plasmid containing the N gene was recombined with in a BaculoDirect™ baculovirus DNA designed in order to express N protein in fusion with a C-terminal polyhistidine tag containing V5 epitope. Sf21 cells were transfected with recombinant baculovirus DNA. Recombinant N protein was extracted from infected cells, analysed by SDS-PAGE and Western blot, and purified by Ni^{2+} affinity procedure. Sera from 100 healthcare workers and five 2–3-year-old children were tested in a Western blot assay using the purified recombinant N protein. All of the sera from adults and two of the sera from children have a positive result.

Keywords: Coronavirus; OC43 strain; Nucleocapsid protein; Baculovirus; Insect cells; Western blot

1. Introduction

Coronaviruses (family *Coronaviridae*, order *Nidovirales*) are enveloped viruses with a linear, non-segmented, positive-sense, single-stranded RNA. The RNA genomes are the largest genomic RNA molecules known to date (27–31 kb). These viruses are divided into three distinct groups named 1, 2, and 3. Five types of human coronaviruses (HCoVs) have been described: HCoVs-229E and OC43 have been recognized since the mid-1960s and belong to groups 1 and 2, respectively. Recently, three other human coronaviruses were discovered. The SARS-associated coronavirus (SARS-CoV) was identified in 2003 during a worldwide epidemic starting from the Guangdong province (Ksiazek et al., 2003; Kuiken et al., 2003). In 2004, another group 1 coronavirus, HCoV-NL63 was reported in the Netherlands (Fouchier et al., 2004; Van der Hoek et al., 2004). In January 2005, a new group 2 coronavirus, HCoV-HKU1, was found in two patients suffering from pneumonia in Hong-Kong (Woo et al., 2005). Most of the seroepidemiological studies on human classical coronaviruses were conducted in 1970s using neutralization on cell cultures, hemagglutination inhibition or complement fixation methods, which are labor intensive and time consuming. As there is no available validated test as yet, it is necessary to develop a serological test for the detection of HCoV-OC43 antibodies to obtain recent data about the seroprevalence of this virus in the population. Development of new serodiagnostic tests requires the use of viral proteins. But the cultivation of HCoV-OC43 is difficult and does not allow for the production of large quantities of viral antigen. The objective here is to describe the expression of a recombinant nucleocapsid protein of HCoV-OC43 in a baculovirus/insect cell system and the development of a Western blot immunoassay for the detection of human antibodies against HCoV-OC43.

2. Materials and methods

2.1. Virus and cells

The cell-line adapted strain of prototype human coronavirus OC43 was obtained from American Type Culture Col-

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lection (ATCC), Rockville, MD. HCoV-OC43 was propagated by inoculation into a 1-day cultivated human rectal tumour cells (HRT18) and incubated for 48 h or 72 h at 35 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium (Gibco™) supplemented with 2% foetal bovine serum, HEPES 20 mM (EUROBIO™), $NaHCO_3$ 0.1% and antibiotics. Propagation of recombinant baculovirus and expression of N recombinant protein were made in *Spodoptera frugiperda* derived Sf21 cell line. Sf21 was maintained in suspension or adherent cultures in Grace's insect cell culture medium (Gibco™) supplemented with 10% fetal bovine serum. BaculoDirect™ C-Term Linear DNA (Invitrogen) derived from *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) DNA was used to build the recombinant baculovirus. It allows transferring gene of interest from the entry plasmid to the baculovirus DNA directly in vitro without the need of recombination in bacterial cells, using specific recombination sites from bacteriophage lambda. The presence of Herpes simplex virus thymidine kinase gene (HSV1tk) and *lac*Z gene located between the two recombination sites allows to inhibit replication of non-recombinant baculovirus in presence of ganciclovir, and to determinate viral purity using β-galactosidase staining (Godeau et al., 1992; Lalumiere and Richardson, 1995). The expressed protein is placed in fusion with a tag containing hexahistidine and V5 epitope sequence (a 14 amino-acid peptide derived from the proteins P and V of the simian paramyxovirus SV5) allowing to detect and purify the recombinant fusion protein Southern et al. (1991).

2.2. Construction of baculovirus entry vector

HCoV-OC43 RNA was extracted from 200 μL of the supernatant of infected HRT18 culture by matrix affinity chromatography with QIAamp® Viral RNA Mini Kit (Qiagen). HCoV-OC43 N gene was amplified by reverse transcriptase (RT)-polymerase chain reaction (PCR) with two custom primers designed from previously reported nucleotide sequence of HCoV-OC43 N gene (GenBank accession no. AY391777) and following industrial guideline instructions: *TMNT*: 5′-CACCATGTCTTTTACTCCTGGTAAG-3′ and *NTHO3*: 5′-TATTTCTGAGGTGTCTTCAGT-3′. Expand High Fidelity PCR System (Roche) was used to amplify the N gene. The amplification product was cloned into pENTR™/D-TOPO® (Invitrogen) and transformed in electrocompetent *Escherichia coli* strain TOP10F' cells. Clones containing the N gene were amplified in LB broth and plasmids were purified with QIAprep® Spin Miniprep Kit (Qiagen). To confirm the insertion in frame and the absence of mutation in the N gene, plasmids were sequenced by BIOFIDAL Society (170 avenue Gabriel Péri, 69120 Vaulx en Velin, France) using M13 forward and reverse primers, and TMNT/NTHO3 primers.

2.3. Construction of the recombinant baculovirus

Recombination reaction was performed 18 h at room temperature in a microcentrifuge tube containing 100 ng (2 μL) of the purified entry vector, 300 ng (10 μL) of the BaculoDirect™ Linear DNA, 4 μL of 5× LR Clonase™ Reaction Buffer and 4 μL of LR Clonase™ Enzyme mix. After incubation time, 2 μL of Proteinase K solution (Invitrogen) was added to the reaction, and incubated 10 min at 37 °C. Lipid mediated transfection of the Sf21 cells was performed with Cellfectin® Reagent (Invitrogen) in six well plates. Each well was seeded with 1.5×10^6 Sf21 cells. Cells were allowed to attach for 1 h at room temperature. Transfection mixture was prepared with 10 μL of LR recombination reaction, 6 μL of Cellfectin® Reagent and 200 μL of unsupplemented Grace's Insect Medium, and incubated at room temperature for 45 min. Medium was removed from each wells and carefully rinsed with unsupplemented Grace's insect medium. Eight hundred microliters of unsupplemented Grace's Insect Medium was added to the transfection mixture and drop onto the cells. Plate was incubated at 26 °C for 5 h. After incubation time, transfection mixture was removed and 2 mL of complete growth media with 10% FBS, antibiotics and 100 μM ganciclovir, was added to each well. Plate was incubated at 27 °C for 72 h in a moisturized box. When the first signs of infection appeared, cell culture medium containing virus was harvested. It was designated as P1 viral stock. To prepare a high-titer viral stock, 500 μL of the P1 viral stock was used to infect 1.5×10^6 Sf21 cells in 1.5 mL of complete growth media with antibiotics and 100 μM ganciclovir. Plate was incubated at 27 °C for 72 h in a moisturized box. When the first signs of infection appeared, cell culture medium containing virus was harvested. It was designated as P2 viral stock. To ensure that non-recombinant virus were eliminated by ganciclovir selection, β-galactosidase staining of three wells containing, respectively, non-infected cells, cells used to produce P1 viral stock, and cells used to produce P2 viral stock, was proceeded using β-Gal Staining Kit (Invitrogen). PCR was used to confirm the presence and orientation of HCoV-OC43 N gene in the recombinant baculovirus. Extraction of total DNA was operated on infected cells using QIAmp® DNA Mini Kit (Qiagen). PCR used for detection of N gene was applied to extracted DNA. Another PCR assay using a combination of a forward primer PHED-F (5′-AAATGATAACCATCTCGC-3′) located in the polyhedrin gene (recommended by Invitrogen) and the reverse primer NTHO3 of the insert was applied to extracted DNA in the same conditions as below.

2.4. Production and analysis of recombinant protein

Sf21 cells in 25 cm^2 flasques were infected with high titer recombinant baculovirus suspension (P2 viral stock) with or whithout 2% FBS. Insect cells and culture medium were harvested 48, 72, 96 and 168 h post-infection. Harvested cells were suspended in 50 mM $NaPO_4$, 500 mM NaCl, pH 8.0 solution, and broken by 10 freeze–thaw cycles using liquid nitrogen and a 37 °C water bath. After centrifugation of cell lysate at 4000 rpm for 15 min, supernatant was stored for SDS-PAGE analysis. Proteins from culture medium and cell lysates were analysed on a 4.8% stacking, 10% resolving polyacrylamide gel by a discontinuous SDS-PAGE system. Precision Plus Protein™ Standards (BioRad) was used as a molecular weight standard. After SDS-PAGE, proteins were transferred onto Trans-Blot® Transfer Medium (Biorad) with Criterion™ Blotter (BioRad).