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Characterization of the Recombinant Proteins of Porcine Circovirus Type2 Field Isolate Expressed in the Baculovirus System

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ABSTRACT

Porcine circovirus (PCV) type2 was isolated using primary porcine kidney cells from lymph node of piglets with typical PMWS. The presence of the virus was identified by PCR using primers specific to PCV type2. The ORFs 1 and 2 were amplified by PCR using primers corresponding to the target genes of the PCV type 2. Cloned genes were inserted into the baculovirus expression vector and PCV recombinant proteins were expressed using baculovirus expression system. Recombinant protein expression was determined by indirect immunofluorescent assay (IFA) and immunoblotting using polyclonal antiserum to PCV. ORF1 gene expressed two proteins with approximately 17 kDa and 31 kDa proteins in the baculovirus system. Recombinant protein of the ORF2 was similar to that of the native virus except minor bands with different molecular weight were detected. Recombinant protein expressed in the baculovirus system showed at least two glycosylation sites based on the tunicamycin treatment. Recombinant protein of the ORF2 assembled virus-like particle in recombinant virus infected insect cells.

Key words : PCV type2, recombinant protein, baculovirus system

Introduction

Post-weaning multisystemic syndrome (PMWS) has been hot issue in swine industry with controversy of causative agent that may produce this newly recognized pig disease. Among several pathogens detected PCV type2 has been consistently identified from animals with typical PMWS (1,2,10,11). However non-pathogenic porcine circovirus (PCV type1) was first detected as a contaminant of the continuous pig kidney cell line PK-15 (13,14). This small non-enveloped single-stranded circular DNA virus has been classified in the circoviridae family (6), along with avian viruses including chicken anemia virus, psittacine beak and feather disease virus (4, 17). The presence of antibodies reactive with PCV1 has also been detected in humans, mice and cattle (16), but challenge experiment to pigs of PK-15 contaminant PCV didn't induce disease (3,15). The viral genomic DNA is very short (approximately 1.7kbp) compare to that of other viruses but it possesses tentatively 11 ORFs by computer analysis (5,9). But major two ORFs of ORF1 and ORF2 have been elucidated their role in the virus (7,8,12). The genomic size of ORF1, encoded rep-associated protein, is 942bp and ORF2, encoded capsid protein, is 699bp, respectively (5). Recently recognized unique PCV is known to associate with pig's new disease, post-weaning multisystemic syndrome (PMWS) worldwide. This PCV named to PCV type2 and 70% of the sequence is homologous compared to PCV type1. In two major ORFs, 83% of ORF1 sequence and 67% of ORF2 is homologous between two strains (11).

In this research, baculovirus protein expression system was used for the expression of the major two ORFs of PCV type 2 known to associate with PMWS in swine. The expressed proteins were characterized with molecular techniques described elsewhere.

Materials and Methods

PCR and cloning

Lymph nodes and tonsils were collected from pigs with PMWS, and DNA was extracted using phenol/chloroform/isopropanol solution. Extracted viral genomic DNA was used as a template to amplifying ORFs 1 and 2 with primers specific to corresponding genes. This amplified PCR product contained open reading frame of the gene encoding the protein associated with viral replication. A primer sequence for the upstream 5′ end of the ORF1 designated as F1 was 5′-ACC AGC GCA CTT CGG CAG-3′ and 3′ reverse primer R1 sequence was 5′-TAA TCC TCC GAT AGA GAG C-3′, respectively. PCR conditions for the amplification was

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as follows. The first one cycle was performed with denaturing for 2 min at 94℃, annealing for 90 sec at 42℃ and extension for 3 min at 72℃. Additional 25 cycles of 90 sec at 94℃, 1 min at 42℃ and 90 sec at 72℃ and final step composed of 90 sec at 94℃, 1min at 42℃ and 10 min at 72℃ was carried out. Amplified gene was cloned into TA vector (Promega T-easy vector system, USA). TA vector containing PCV type2 ORF1 was digested with EcoRI and subcloned into CIAP treated baculovirus transfer vector pVL1393 (Invitrogen, USA).

The PCV type2 ORF2 was amplified with 700bp full sequence. Primer set was F2 : 5′-TTT AGG GTT TAA GTG GGG GGT C-3′ and R2 : 5′-ccggatcc ATG ACG TAC CCA AGG AGG CG-3′. This primer set had minor modification from the one described by Nawagitgul et al. and reverse primer was included BamHI restriction site for cloning (12). PCR conditions for the amplification consist of denaturation step at 95℃ for 5 min, and additional 30 cycles of denaturation at 94℃ for 1min, annealing at 53℃ for 1 min and extension at 72℃ for 1 min. PCR was carried out using thermocycler (Perkin Elmer, USA). PCR product was cloned into baculovirus expression vector pVL1393 as same manner as ORF1.

Sequencing

To confirm proper start and stop codons cloned DNAs of ORF1 and ORF2 of the PCV type2 were sequenced by Sanger's method using automated sequence analyzer (Bioneer Co, Ltd., Korea).

Transfection

Hi-five cells were maintained with EX-cell 400 (JRH scientific, USA) with 1% of antibiotics and antimycotic solution (Gibco-BRL, USA). Insect cells were seeded into 6 well plate and placed 25℃ for 2 hrs. Transfection mixture containing linearized baculovirus DNA (Baculogold, Phamingen, USA) and pVL1393 vector with insertion of PCV ORF 1 or 2 genes were mixed with Lipofectin (Gibco-BRL, USA) and was placed in room temperature for 10 min. And cells were transfected with transfection mixture drop by drop and placed at room temperature. After 6 hrs of incubation, the medium using transfection was removed and replaced with 5ml fresh insect cell culture medium Ex-cell 400.

After 72 hrs of incubation at 25℃ supernatant was collected and the cell plate was air dried and fixed with methanol at -20℃ for 15 min. To determine expression of the recombinant protein from transfected cells by IFA using polyvalent antiserum was carried out.

Immunoblotting

Hi-five cells ($5X10^6$) were seeded into $75cm^2$ plastic cell flask and 2 hrs later recombinant baculovirus was inoculated and placed at 25℃ incubator for 90min. After 72 hrs of infection, when obvious CPE was observed, cells and supernatant were harvested. Control cells and supernatant were mixed with 2X treatment buffer, boiled at 100℃ for 5 min, placed on ice and then centrifuged at 12,000 rpm for 1min. Samples were electrophoresed in 10% SDS-PAGE gel and proteins were transferred onto PVDF membrane using electrotransfer system. Membrane was blocked with 5% skim milk, and reacted with 1:100 diluted polyvalent anti-PCV antiserum for two hours, and washed three times with PBS-Tween. HRP-labeled goat anti-swine secondary antibody (KPL, USA) was reacted for one hour to capture the recombinant protein bound swine IgG. The membrane was washed and agitated into chemiluminascence(Intron, Korea) for 1 min. Then the membrane was exposed to X-ray film and developed.

Tunicamycin treatment

Insect cells were seeded into $75cm^2$ cell flask and 2 hrs later recombinant baculovirus was inoculated and placed at 25℃ for 90 min. The inoculums were replaced with fresh insect cell culture medium Ex-cell 400 including 1μg/ml tunicamycin. After 72 hrs of incubation, cells were harvested and immunoblotted by same manner with previous protein analysis using SDS-PAGE.

Virus-like particle formation

VLP formation was determined by electronmicroscopy (Jeol, Japan) with negative staining using 2% PTA. Recombinant virus infected insect cells was frozen and thawed three times and supernatant was collected. Insect cell culture supernatant was centrifuged and pellet was resuspended with distilled water for the electronmicroscopy.

Results

ORFs 1 and 2 of the PCV type2 have been amplified by PCR using primer set corresponding to target gene with expected genomic size. The PCR products was purified using Geneclean II kit (Bio101, USA) for the expression vector construction. Each of the ORF was appropriately ligated into the baculovirus expression vector pVL1393 and confirmed with restriction endonuclease digestion followed by agarose gel electrophoresis with ethidium bromide staining. Cloned DNA sizes were similar to that of the expected in agarose gel electophoresis. Baculovirus expression vector (lane 2) and ORFrs 1(lane 3) and 2(lane 4) were released using appropriate restriction endonucleases (Fig. 1). Recombinant baculoviruses containing PCV type2 ORF1 or ORF2 were generated from linearized baculovirus DNA and baculovirus construction vectors transfected insect cells. PCV type2 recombinant proteins were successfully expressed from recombinant virus infected insect cells. Expression of the recombinant proteins from recombinant virus infected insect cells were confirmed by IFA test using polyclonal antiserum to PCV type 2. Intracytoplasmic fluorescence was detected from recombinant DNA transfected insect cells but from vector alone transfected nor control cells (Fig. 2).

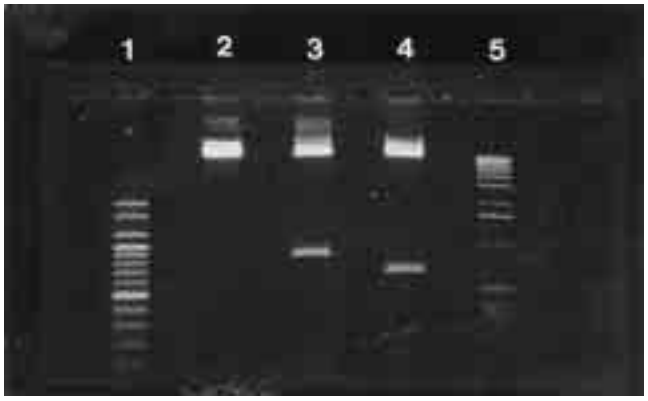

Fig. 1. Cloning of the ORF 1 and 2 of the PCV type2 in pVL 1393. Lanes 1 and 5 show DNA size markers of 100bp and 1Kb. Lanes 2, 3 and 4 indicate pVL1393, pVL1393 inserted with ORF1 and pVL1393 with ORF2, respectively.

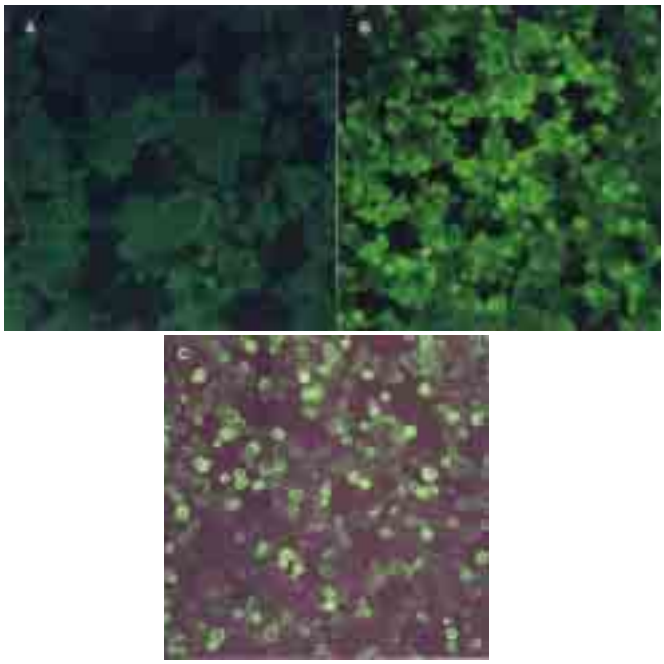

Fig. 2. Indirect immunofluorescent test using polyclonal antibody to PCV. A showing no positive immunofluoresce whereas B showing strong positive reaction in the cytoplasm of the PCV ORF1 gene inserted recombinant baculovirus infected insect cells. The PCV ORF2 gene containing recombinant baculovirus infected insect cells showed intracytoplasmic fluorescent with polyclonal antiserum (C).

Immunoblot analysis

The ORF1 of the PCV type2 has been expressed as lane 2 shown in Fig. 3. One high molecular weight band and another fast migrating small molecular weight band (large open arrow head) were detected with polyvalent antiserum. The ORF2 of PCV type2 recombinant virus expressed proteins with several different molecular masses (Fig. 3, lane 3 and 4). Major protein band was comparable to that of native virus and two minor proteins were simultaneously expressed. The recombinant proteins treated with tunicamycin did not change molecular mass of the major band but two minor bands were shifted as shown in lane 4 (small open arrow heads). This may indicate that the ORF2 of the PCV has minor glycosylation modification or insect cell expression system does not mimic porcine cell translation system.

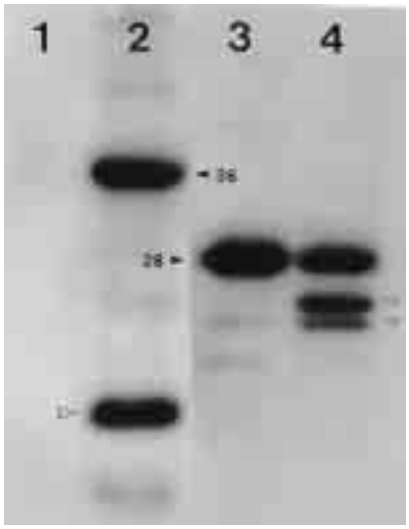

Fig. 3. Western blot analysis of the porcine circovirus recombinant proteins. Lane 1 represents insect cell lysate and lane 2 shows recombinant PCV type2 ORF1 recombinant proteins with high (approximately 36kDa) and low molecular weight (17kDa), respectively. Lane 3 is a recombinant protein of the ORF 2 and lane 4 represents recombinant ORF2 with tunicamycin treatment.

VLP formation

Electronmicroscopy revealed recombinant baculovirus containing ORF2 of the PCV2 infected insect cells were released virus-like particles. Icosahedral form of virus-like particles were detected from ORF2 recombinant virus infected insect cells but ORF1 infected cells(Fig. 4).

Discussion

Among new emerging swine diseases PMWS became one of a most controversial diseases. PCV type2 has been speculated as a major causative agent of the PMWS in pigs. However there is no clear evidence that the virus causes PMWS except PCV was detected from majority of the pigs showing clinical signs. Since PCV replicates in the secondary immune system it has been raised great concern on the xenotransplantation using pig as an animal model. Immunocompromised individual may cause life threatening serious problem when organs transplanted into the human recipient from genetically modified pig donors infected with PCV. So far known molecular characteristics of the PCV

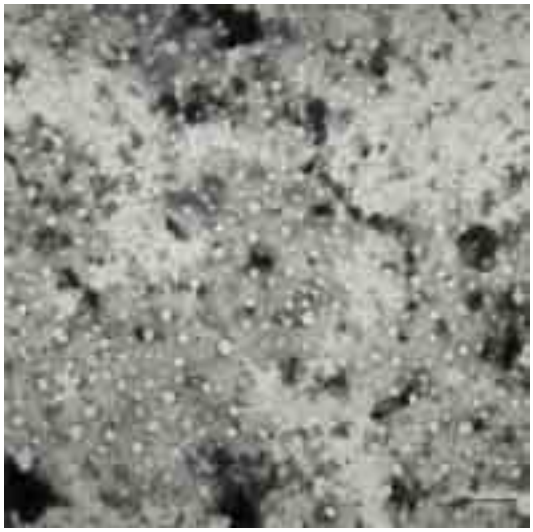

Fig. 4. Electronmicrograph of the PTA stained PCV ORF2 recombinant proteins expressed in the baculovirus system showing virus-like particles. Majority of VLP was similar to that of the native PCV particles but size of particles was heterogeneous. Bar represents 100 nm in length.

type 1 and 2 are similar to each other with genetic organization and size of the viral genome. But the instead of the fairly small viral genome size genetics and function of the each ORF of the PCV is not fully understood at the moment. The ORF2 of the PCV type2 showed heterogeneity compare to that of the ORF2 of the PCV type1 (5).

Putative protein sequence of the PCV ORF2 possessed at least a glycosylation site but major recombinant protein expressed in the baculovirus system did not show any noticeable changes in the molecular mass by tunicamycin treatment. This may indicate that the ORF2 of the PCV has minor glycosylation modification or insect cell expression system does not mimic porcine cell translation system. The post translational modification of the recombinant ORF2 need to be further characterized to understand nature of the ORF2 in the PCV type 2. At the moment we do not understand two minor bands appeared to be shifted from major band after tunicamycin treatment.

The recombinant proteins expressed in the eukaryotic expression system could be utilized as a diagnostic antigen, immunogen for the recombinant vaccine and clue to understand molecular characteristic of the viral protein. Further research of the recombinant protein such as immunogenicity comparison with native viral protein, function of the each recombinant protein in the pathogenicity and viral replication would be beneficial to solve the PMWS in pigs. Currently immunogenecity of the recombinant protein is under testing in the host animals to understand role of the recombinant protein in sows and piglets received maternal antibody from vaccinated sows.

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