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Identification of porcine circoviruses with genetic variation from lymph nodes collected in pigs with PMWS

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Abstract : New emerging post weaning multisystemic wasting syndrome (PMWS) threatening swine industry worldwide and investigation of the etiological agent is underway. The porcine circovirus (PCV) consistently identified from PMWS pigs and research result indicate that there is strong relationship between PCV and PMWS. Farms with PMWS submitted pigs suffered from various PMWS typical signs and necropsy finding showed lymph node anomalies. The PCV DNA was amplified from inguinal lymph node collected from pigs with PMWS. PCV specific primers were successfully amplified PCV DNAs and were able to differentiate PCV type I and II. We have identified noble PCV virus with genetic variation. The virus showed insertion of the nucleic acid at the 5' of the genome but did not have PCR product with primer set corresponding to PCV type II virus.

Key words : PCV, PCR differentiation.

Introduction

Post-weaning multi-systemic wasting syndrome (PMWS) has been drawn attention as a putative causative agent for the PMWS in young pigs[1~15]. Porcine *circovirus* was consistently found in PMWS pigs as well as PK-15 cell lines[1~3, 16~20] and molecular characteristics of the virus was elucidated. But etiologic association of the PCV with PMWS is still controversial because of ubiquitous presence of the virus in the pig populations. The PCV belongs to a *Circoviridae* along with chicken anemia virus (CAV) and psittacine beak and feather disease virus(PBFDV)[5,16~18,21~23]. PMWS pigs aged from 5 to 8 weeks showed clinical signs include wasting syndrome, dyspnea, unthriftness, lymph node enlargement, anemia and icterus had *circovirus* infection but no clear evidence has been provided that the porcine circovirus caused these syndrome. Porcine circovirus[17~

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[18,21,24-25] belongs to *Circoviridae* containing single stranded circular DNA with 1.7kb in size. The virus is non-enveloped and 17nm in diameter. Pigs with poor performance after weaning were submitted to the immunopathology Lab. Konkuk University. Pigs had prominent inguinal lymph node enlargement and hyperemia but no obvious gross changes in other organ have been found at the necropsy[3,4,10, 24,26]. The farms submitted these pigs found sero-positive to PRRSV and swine influenza without previous vaccination[3-8]. We have been able to demonstrate two different types of PCV by polymerase chain reaction (PCR)[6,11,27,28] and these two PCVs were distinguishable from PK-15 cell derived PCV type I.

Materials and Methods

Indirect immunofluorescent assay : Sero-epidemiological assay was carried out to determine prevalence of the antibody to porcine circovirus using PK-15 cells, which persistently infected with porcine circovirus. Presence of the circovirus in PK-15 cells confirmed by indirect immunofluorescent test using polyvalent antibody to PCV. Briefly, cells were seeded into 96 well cell culture plates and incubated for 96 hours with 5% CO_2. Cells were fixed overnight at 5℃ with cold fixation solution containing 50% methanol and 50% acetone. To eliminate false positive reaction by porcine parvovirus contamination, which is occasionally found in cell culture, indirect immunofluorescent test was carried out using monoclonal antibody specific to PPV. Total DNA was isolated from lymphoid tissue and PK-15 cells using Genomic DNA purification system (Promega USA). Briefly, lymphoid tissue was homogenized in liquid nitrogen and DNA was prepared followed by manufacturer's recommendation

PCR amplification of the PCV specific genomes : A PCR condition for the amplification of the PCV DNA was optimized using genomic PCV DNA extract from persistently infected PK-15 cells. Primer sets for the PCR amplification were designed based on the sequences downloaded from Genbank (Fig 1)[1,3,18-19]. PCV specific DNA was amplified by PCR reaction using primer set corresponding to PCV type I and II. The internal primer specific to PCV type II was designed for the differential diagnosis. Non-pathogenic PCV type I could be amplified using primers F1 and R1 combination and internal F2 primer was designed to amplify DNA from potentially pathogenic type II. PK-15 cell culture fluids and lymph nodes submitted to the laboratory were used for the PCR. Primer sequences for the extreme 5' end was F1 : 5'-ACC AGC GCA CTT CGG CAG-3' and internal primer sequence for the distinguishing type I and type II was F2 : 5'-TGA GTA CCT TGT TGG AGA GC-3' and common 3' reverse primer sequence was R1 : TAA TCC TCC GAT AGA GAG C-3'. To amplify the specific genomic DNA of the PCV lymph nodes collected from PMWS piglets were homogenized in liquid nitrogen and extracted DNA using Progema (USA) kit followed by manufacturer's protocol. PCR conditions for the amplification was as follows the first one cycle was performed with denaturing step of 2 min. at 94℃, 90 sec at 42℃ and 3 min at 72℃. Additional 25 cycles of 90 sec at 94℃, 1 min at 42℃ and 90 sec at 72℃ final step composed of 90 sec at 94℃, 1 min at 42℃ and 10 min at 72℃ was carried out.

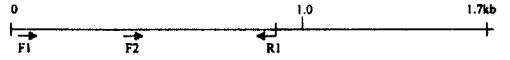

Fig 1. Schematic diagram of the PCV genomic DNA and arrows represent positions of the primers designed for the PCR.

Results

Indirect immunofluorescent assay : To identify an infection of the PCV type I in PK-15 cell line indirect immunofluorescent assay was performed[3,19,29,30]. PCV infected PK-15 cells showed PCV specific intra-nuclear immunofluorescence reaction (Fig 2). Positive reaction was appeared at the marginal region of the nucleus and distinct focal granule type of the positive reaction was detected. But these cells did not showed positive reaction to monoclonal antibody against porcine parvovirus. PK-15 cells showed positive reaction with persistent infection of the PCV type I and

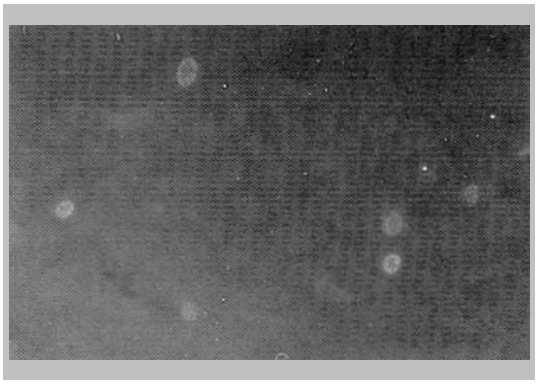

Fig 2. PCV viral antigen was detected by indirect immunofluorescent test from PK-15 cells using polyclonal antibodies to PCV. A few positive intra-nuclear focal immunofluorescent reaction of the PCV in PK-15 cells evident from uninfected cells on the dark background.

used for the sero-epidemiology of the PCV infection using serum samples collected from swine industry in mid west region of the Korea. Sero-positive rate to the PCV type I in swine industry was varies and around 10% of the tested serum samples were positive but did not tested against PCV type II.

PCR amplification of the circovirus specific DNA : Primers F1 and R1 corresponding to the 5' prime 886bp were efficiently amplified PCV from PK-15 cells (Fig 3)

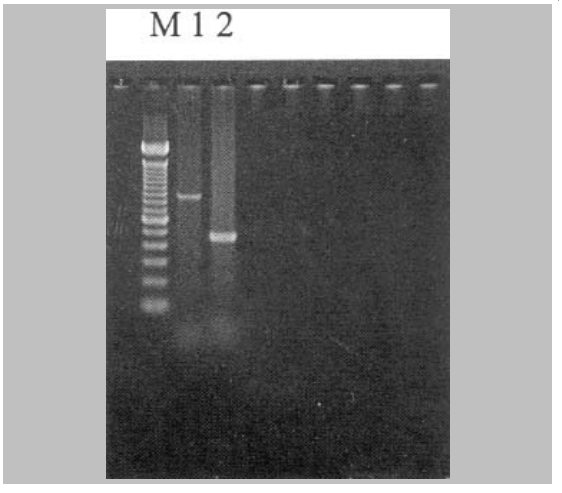

Fig 3. Optimization of the PCR for the amplification of the PCV DNA using primers corresponding to PCV type I and II. Primers F1 and R1 was specific to PCV type 1 with DNA product of 886bp (lane 1) from PK-15 cells. PCR amplified 469bp product was derived from primer F2 and R1 specific to type II virus (lane II) using lymph node of a PMWS pig.

and inguinal lymph node preparations (Fig 4). Sample designated IC98 showed PCR product of 886bp which is same migration pattern in agarose electrophoresis with PCV type I PCR product from PK-15 cells but DB99 had heavier migration compare to that of PCV type I from PK-15 cells and IC98. Primer set containing F2 and R1 amplified 469bp product from IC98 but from PK-15 cells and DB99 (Fig 4).

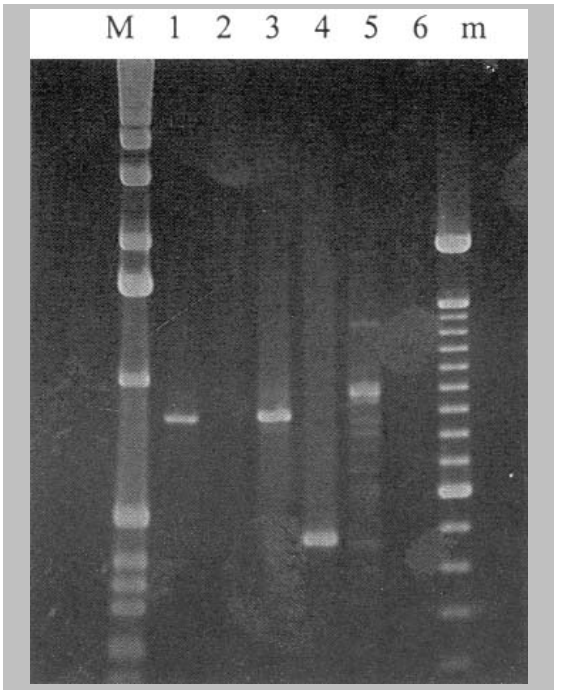

Fig 4. PCR product from PK-15 cells and lymph nodes collected from PMWS piglet. PCR product of 886bp was amplified from PK-15 cells (lanes 1,2) and IC98 (lanes 2,3) and heavier product with approximately 80bp insertion was observed from DB99 (lanes 5,6) using primers F1 and R1. Primers F2 and R1 amplified only IC98 but PCV DNA derived from PK-15 cells and DB99 lymph node. M : 1kb DNA size marker, m : 100bp DNA size marker.

There are two different migration patterns might suggest that a presence of more than 1 types of PCV in swine industry in Korea.

Discussion

The porcine circovirus as a controversial etiological agent has been consistently isolated from PMWS pigs. There is no

clear evidence that this virus is the causative agent for the PMWS but research data indicate that there is a strong relationship between PMWS and circovirus in pigs[9,12,31]. Several different strains of the virus have been isolated from various regions of the world and their genomic organization has been characterized. Morozov *et al*[27] isolated the noble PCV virus with insertion of the nucleic acid in the PCV genome. PCV DNA was successfully amplified from lymph node collected from pigs with PMWS by PCR using primers specific to PCV type II. The IC98 showed two distinct PCR products with expected size on an agarose gel electrophoresis. But DB99 showed heavier migration of the 886bp PCR product which is homologous domain of the PCV type I and II. But there is no 469bp band, which presumably specific to PCV type II, was detected with primer set designed for the differentiation of the PCV type I and II. This study suggests that these primer set can be used for the differentiation of the type I and type II but PCR amplification of PCV DNA with these primers may not differentiate pathogenic PCV from non-pathogenic PCV. In this study PCVs with PMWS from different regional background showed various migration patterns in agarose gel electrophoresis. The size of the insertion was different from previous publication[11,18,23,25,27]. Also, the result suggests that there are more than one types of PCV present in swine industry in Korea. These viruses need to be characterized by means of molecular biologically and pathogenically in host animals. Porcine parvovirus free PK-15 cells showed positive reaction to polyclonal antibody to PCV and successfully employed for the serological investigation of the PCV infection in pigs. Limited numbers of serum sample were analyzed for the presence of the antibody to PCV and 10~20% of the field samples showed sero-positive to PCV type I using PK-15 cells with persistent infection of the PCV type I virus[2-4]. But these sero-positive rates may not represent serological statue of the pigs in the field with pathogenic PCV type II infection. Isolation of the field strain and serological survey using the strains prevalent in the farm need to be carried out to understand field situation of the PMWS in Korea. Pathogenicity of the Korean strains of PCV need to be further investigated along with their virulence and molecular characteristics. PCR amplification of the PCV DNAs from PMWS pigs and PK-15 cells indicate that there are porcine circovirus present in pigs with clinical signs. The virus DNA from PMWS pigs were differentially amplified using primer set specific to PCV type II and it was similar result that of papers published elsewhere. The DB99 pig were showed no amplifications of the DNA using primers corresponding to the type II virus but product with primer set designed to amplify the PCV type I yield larger molecule compare to that of prototype strain of PCV from PK-15 cells. This result indicates that there are more than one type of the PCV strain exist in the field and remain to be scrutinizing their pathogenicity and a role of PCV in PMWS pigs.

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