บทคัดย่อ

ทำการศึกษาแผนผัง epitopes ของ capsid protein (ORF2) ของ porcine circovirus type 2 (PCV2) โดยการใช้ โมโนโคลนอล แอนติบอดี้ ที่เฉพาะ จำนวนเจ็ดโคลน ในการตรวจหาตำแหน่งที่เป็นแอนติเจน บน capsid protein ของไวรัสลูกผสม ระหว่าง PCV1และ PCV2 ไวรัสลูกผสมสร้างจากการตัดส่วนของ PCV2-ORF2 เป็นลำดับ และเชื่อมส่วนที่ถูกตัดด้วย ORF2-PCV1 จากนั้นทำการ transfect ไวรัสลูกผสม PCV1/PCV2 เข้า PK-15 cells และทดสอบการมีชีวิตของไวรัสลูกผสมโดย immunofluorescent assay (IFA) กับ convalescent PCV2 swine antiserum ไวรัสลูกผสมที่กรดอมิโน จำนวน 47 หน่วยขาดหายไปจาก ปลายด้าน NH_2 สามารถจับกับ โมโนโคลนอล แอนติบอดี้ ทั้งเจ็ดโคลนได้ดี เมื่อทำให้กรดอมิโนที่ปลาย NH_2 ของ PCV2-ORF2 ขาดหายไปจาก 47 เป็น 57 (r175) เป็นผลให้ไวรัสลูกผสม ไม่สามารถจับกับโมโนโคลอนอล แอนติบอดี้ 3B7, 3C11, 6H2 และ 8F6 ได้ ไวรัสลูกผสมที่มีกรดอมิโนที่ปลาย NH_2 ของ PCV2-ORF2 ขาดหายไป 62 หน่วย ไม่ สามารถจับกับ โมโนโคลนอล แอนติบอดี้ ทั้งเจ็ดโคลนได้ ถ้ากรดอมิโนลำดับที่ 165 ถึง 233 ที่ปลายด้าน COOH ของ PCV2-ORF2 ถูกแทนที่ด้วยลำดับกรดอมิโนของ PCV1-ORF2 ไวรัสลูกผสมดังกล่าวไม่สามารถจับกับโมโนโคลนอล แอนติอดี้ ทั้งเจ็ดโคลน ได้ เมื่อยืดลำดับกรดอมิโนของ PCV2-ORF2 จากหน่วยที่ 165 (r464) เป็น 185 (r526), 200 (r588) หรือ 224 (r652) ทำให้ไวรัสลูกผสมสามารถจับกับ โมโนโคลนอล แอนติบอดี้ 3C11, 6H2, 9H7 และ 12G3 ได้ เมื่อกรดอมิโนจำนวนสี่ หน่วยที่ปลายด้าน COOH ของ r588-ORF2 ถูกแทนที่ด้วย กรดอมิโนของ PCV2-ORF2 ทำให้ได้ไวรัสลูกผสม r588F ซึ่ง สามารถจับกับ โมโนโคลนอล แอนติบอดี้ ทุกโคลนได้ ผลที่ได้ชี้ให้เห็นว่า โมโนโคลนอล แอนติบอดี้ ทั้งเจ็ดโคลน จดจำ epitopes จำนวน อย่างน้อยห้าตำแหน่ง บน capsid protein ของ PCV2 ซึ่ง epitopes เหล่านี้น่าจะซ้อนทับอยู่ภายในช่วงกรดอมิโนลำดับที่ 47 ถึง 63, 165 ถึง 200 และ กรดอมิโน สี่หน่วยสุดท้ายทางปลาย COOH ของ capsid protein ของ PCV2 epitopes ทั้งห้านี้ น่าจะเป็น conformational epitopes ซึ่งอยู่บนผิวภายนอกของอนุภาคไวรัส

Abstract

Seven monoclonal antibodies (MAb) against the capsid protein of type 2 porcine circovirus (PCV2) were used to map the antigenic sites using PCV2 infectious DNA clones containing PCV1/PCV2- open reading frame (ORF) 2 chimeras. The chimeric PCV1/PCV2-ORF2 cassettes were constructed, by serial deletions of the PCV2-ORF2 and substitution of the deletions with the corresponding sequences of the PCV1-ORF2. The viability of the chimeras in transfected PK-15 cells was confirmed with a convalescent PCV2 swine antiserum. The chimeric PCV1/PCV2 clones were transfected into PK-15 cells, and their reactivities with the seven MAbs were detected by immunofluorescent assay (IFA). The chimera with a deletion of 47 amino acids at the N-terminus of PCV2-ORF2 (chimera r140) reacted strongly to all seven MAbs. Expanding the deletion of PCV2-ORF2 from amino acid residues 47 to 57 (r175) abolished the recognition of MAbs 3B7, 3C11, 4A10, 6H2 and 8F6 to the chimera. Further deletion of PCV2-ORF2 to 62 residues disrupted the binding of this chimera to all seven MAbs. Reactivities with all MAbs were absent, when amino acid residues 165-233 at the C-terminus of ORF2 was replaced with the corresponding PCV1-ORF2 sequence. Extending the sequence of PCV2-ORF2 from residues 165 (r464) to 185 (r526), 200 (r588) or 224 (r652) restored the ability of the three chimeras to bind MAbs 3C11, 6H2, 9H7 and 12G3 but not MAbs 8F6, 3B7, or 4A10. When the four amino acids at the C termini of r588 were replaced with that of PCV2-ORF2, the resulting chimera (r588F) reacted with all seven MAbs. The results from this study suggest that these seven MAbs recognized at least five different but overlapping epitopes within residues 47 to 63, 165 to 200 and the last four amino acids at the C-terminus of the PCV2 capsid protein. These epitopes are likely conformational epitopes on the exterior surface of the capsid protein.

-Cloning materials (Competent cells for subcloning, T4 DNA ligase, Restriction enzymes, Bacto Agar, Yeast extract, IPTG, β-Gal, etc.) 20,000

-Kits (DNA purification kit, Plasmid miniprep kit, ect.) 15,000

-Transfection reagents (Lipofectamine2000) 10,000

-Immunological reagents (anti-mouse IgG FITC conjugate, anti-rabbit IgG FITC conjugate,) 16,000

-Media (MEM, FBS, antibiotic-antimycotic, Trypsin, HBSS, etc.) 35,000

-Chemical reagents and ultra-pure water 28,000

-Rabbits, husbandry and care 15,000

3. Publication cost and office materials 26,000

Total 247,000

Execcutive Summary

1. ปัญหาที่ทำการวิจัย ความสำคัญและของปัญหา

Porcine circovirus (PCV), classified in the family *Circoviridae*, is a small non-enveloped DNA virus. There are two genotypes of PCV, the non-pathogenic strain-PCV1 and the pathogenic strain-PCV2. PCV2 was isolated from pigs with postweaning multisystemic wasting syndrome (PMWS), a recently emerged disease characterized by progressive weight loss, emaciation, difficult breathing and jaundice. PMWS has been recognized as one of the important diseases of swine, and PCV2 infection has been reported worldwide, including Canada, the United States, Europe and Asia. In addition, PCV2 was also isolated from PMWS cases in Thailand. The disease frequently occurs in nursery or grower pigs. Morbidity is usually low, but case fatality can be higher than 50% in epidemic herds. As the impact of PMWS to swine industry has increased dramatically, the World Health Organization for Animal Health (Office International des Epizootics-OIE) is considering PMWS to the list of pig diseases that must be declared internationally by infected countries.

While PMWS and PCV2-related diseases increase threat in swine population throughout the world, an effective mean of prevention and control has not been established. **Our long-term goal** is to develop effective strategies for prevention and control of PMWS and PCV2-related diseases. Therefore, information regarding this virus is required to the understanding of its interaction with the host. Securing background information related to viral coat/capsid protein(s) is essential, because coat proteins play an important role in biological activities such as binding to cellular receptors/ligands for infection and eliciting immune responses. The latter function suggests the possible use of the coat/capsid protein(s) for vaccine development and diagnostic purpose.

2. วัตถุประสงค์

The goals of this study are to map antigenic determinants of the major capsid protein of PCV2 using PCV1/PCV2 chimeras and characterize the chimeras biologically. We hypothesize that the major capsid protein of PCV2 contains neutralizing epitope(s), and the PCV1/PCV2 chimeric capsid protein containing neutralizing epitope is able to protect PCV2 infection. To accomplish these objectives, we will pursue three specific aims:

1. Map antigenic epitopes on the major capsid protein of PCV2 using PCV1/PCV2 chimeras.
2. Examine infectivity of PCV1/PCV2 chimeras in *vitro*.

Test ability of the PCV1/PCV2 chimera containing the neutralizing epitope to induce neutralizing antibodies.

3. ระเบียบวิธีวิจัย

1. Map antigenic epitopes on the major capsid protein of PCV2 using PCV1/PCV2 chimeras.

Chimeric ORF2 cassettes containing ORF2 segments of PCV1 and PCV2 will be constructed by PCR using an elongase enzyme (Gibco BRL) for high fidelity amplification. The templates for amplification are the prototype PK-15 cell contaminant, PCV1, and PCV2 ISU31. PCR will be performed using pairs of internal primers, each containing an overlapping sequence of approximately 10 to 20 nucleotides at the recombination junction. The joining sites will be determined according to the homology of ORF2-PCV1 compared with ORF2-PCV2. Primers at the 3' and 5' ends of the ORF2 gene will be similar to those used for amplification of ORF2-PCV1 or ORF2-PCV2 and will be engineered to contain *Xho*I and *Hind*III sites for cloning.

The remaining PCV2 genomic sequence, called the ORF1-PCV2 fragment (containing ORF1 and non-coding regions), will be amplified with primers flanked with *Bam*HI and *Xho*I sites at the 5' end, and a *Hind*III site at the 3' end. Both ORF2 cassettes and the ORF1-PCV2 fragments will be cloned into plasmid pKSII+ (Stratagene) at *Xho*I-*Hind*III and *Hind*III-*Bam*HI sites, respectively. As a result, the insert consists of a full length PCV genome flanked by *Xho*I sites. The integrity of the base composition of the constructs will be verified by sequence analysis.

To test reactivities of each chimera with MAb, the chimeras will be excised with a restriction enzyme, *Xho*I, to create *Xho*I fragments containing the entire sequence of the PCV2 or PCV chimeras (Fig. 1). One microgram of the purified *Xho*I fragments will be self-ligated at at room temperature for one hour using T4 DNA ligase (Gibco BRL). The ligation conditions will be optimized to facilitate the formation of a circularized DNA containing only a single genomic copy resembling the natural PCV replicative form rather than a multi-genomic copy. The self-ligated *Xho*I fragments will be transfected into PK-15 cells grown overnight using Lipofectamine2000 (Gibco BRL) according to the manufacturer's instructions. At 24 hours

post-transfection, cells will be fixed with absolute methanol and used for immunofluorescent assay with the polyclonal antibodies or MAbs.

2. Examine infectivity of PCV1/PCV2 chimeras in vitro.

The circularized chimeric PCV1/PCV2 clones will be transfected into PK-15 cells as described previously. At 72 hours post transfection, the transfected cells will be washed before frozen and thawed three times. The transfected cell suspension will be clarified and the supernatant will be use for inoculating PCV-free PK-15 cells. The process will be repeated at a total of three times. The third passage of the chimeric PCV will be tested for the present of the infectious virus by immunofluorescent assay. The genomic DNA of each infectious chimera will be isolated, amplified and sequenced to verify the integrity of base composition. The clone that showed positive results will be called infectious clone.

3. Test ability of the PCV1/PCV2 chimera containing the neutralizing epitope to induce neutralizing antibodies.

PCV1/PCV2 chimera reacting with neutralizing MAb(s) will be propagated in PK-15 cells and purified by isopycnic centrifugation. Each purified chimeric PCV will be inoculated into 2 rabbits at a total of three times with 3 weeks interval to produce polyclonal antibodies to the chimeras. The antibodies will be examined for viral neutralization activity using a foci reduction assay. Viral neutralizing activity is the ability of antibodies to reduce the number of positive foci by more than 80% when compared with the control.

4. Time-table with six months interval

Tentative accomplishment	Period within the first year		Period within second year	
	1-6	7-12	1-6	7-12
1. Construct series of chimeric ORF2 using PCR techniques	←	→		
2. Clone ORF2 cassettes into plasmid pKSII+		←	→	
3. Subclone ORF1 and non-coding region into plasmid pORF2		←	→	
4. Sequence insert of the each selected clone			←	→
5. Produce self-ligated PCV chimeric clone			←	→
6. Test reactivities between each PCV chimera and MAbs			←	→

Tentative accomplishment	Period within the first year		Period within second year	
	1-6	7-12	1-6	7-12
7. Test infectivity of each PCV chimera	←	→		
8. Propagate selected PCV chimera containing neutralizing epitope(s) in PK-15 cells and purified the PCV chimera to prepare antigen for inoculation			←	→
9. Produce rabbit hyperimmune sera using purified PCV chimera as antigens			←	→
10. Test the rabbit sera for antibodies to PCV2			←	→
11. Test the rabbit hyperimmune sera for neutralizing antibodies to PCV2 by foci reduction assay			←	→
12. Prepare manuscripts for publication in international journal(s)				← →
13. Prepare report for TRF				← →

5. Topic to be published in international journal

1. Epitope mapping of the major capsid protein of porcine circovirus type 2 using PCV1/PCV2 chimeras. To be published in journal of virology
2. Chimeric porcine circovirus (PCV) types 1 and 2 induce neutralizing antibodies against PCV2. To be published in journal of general virology

6. Budget

1. Salary: Monthly salary for researcher 10,000 X 24 = 240,000 bahts
2. Equipments and materials:

-Disposable plastic ware (cell culture flasks, plastic tubes, microfuge tubes, pipette tips, transfer pipettes, polyallomer tubes, syringes, needles, ect.) 42,000

-PCR and gel electrophoresis related materials (Primers, Taq polymerase, Elongase, 1 kb plus, Agarose IV, ect.) 22,000

-Sequencing expense 18,000