Generation of Plasmids Expressing ORF1-4 of PCV2 as a Novel Research Focus

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Abstract

Porcine circovirus (PCV) is a single-stranded, non-enveloped, DNA virus with an unsegmented circular genome. PCV type 2 (PCV2) infection is widespread and essentially all pig herds are infected with PCV2; however, few have PCV2-associated diseases (PCVAD). The virus is of great concern due to its impact on the farming industry and potential to become zoonotic. Because it is the common link among disease, it is vital to understand the biology of this virus for disease control. This project involved designing primers for the four major open reading frame (ORFs) of the viral DNA, amplifying the sequences of interest using PCR, cloning the ORFs into mammalian expression vectors, and sequencing the cloned material. In the future, this project aims to isolate the purified viral protein encoded by the ORFs and develop antibodies for laboratory use that are not commercially available.

Introduction

Porcine circovirus (PCV) is a 1.7 kb, single-stranded, non-enveloped, DNA virus with an unsegmented circular genome. PCVs are the smallest known viruses that replicate autonomously in the nucleus of eukaryotic cells. It uses the host polymerase for genome amplification. There exist two phenotypically different but genetically related strains of PCVs, PCV1 and PCV2 (Gillespie, 2009). PCV1 has no pathogenicity, but PCV2 is involved in a number of pig diseases (Lou, 2011). The genome of PCV2 is predicted to contain as many as 11 open reading frames (ORFs). The are four major ORFs—ORF1, ORF2, ORF3, and ORF4. The first encodes two proteins involved in DNA replication, and the second encodes a capsid protein. The third is predicted to encode an apoptosis-inducing gene, while the fourth, which is embedded in the third, is predicted to encode an apoptosis-suppressing gene (Ren, 2016).

PCV2 is currently considered one of the most critical viral pathogens of the United States pig population. It is found worldwide, although the origin is unknown. The virus infects a variety of pig cell types, including hepatocytes, cardiomyocytes, and macrophages, although it was first recognized as a contaminant of the porcine kidney cell line PK-15 (Opriessnig, 2007). Infection of PCV2 leads to lymphoid depletion, histiocytic infiltration, and immunosuppression. It can influence several cellular processes such as signaling pathways, miRNA expression, and oxidative stress. Some of these responses increase viral proliferation, while other responses inhibit viral proliferation (Ren, 2016).

PCV2 is the primary causative agent of several syndromes collectively known as porcine circovirus-associated disease (PCVAD) (Gillespie, 2009). One example of a PCVAD is PCV2 related porcine dermatitis and nephropathy syndrome (PDNS). The most obvious sign for this is the development of skin lesions; in more intense cases the pigs show signs of depression,

hypothermia, and weight loss (Drolet, 1999). Another disease syndrome is PCV2 related post-weaning multisystemic wasting syndrome (PMWS). The physical symptoms range from dyspnea, enlarged lymph nodes, pallor, diarrhea, and jaundice. Morbidity and mortality are associated with PMWS (Allan, 2000). A study from 2013 mentioned that PMWS was one of the most economically damaging diseases for the pig industry worldwide in the last 15 years (Alarcon, 2013). The economic impact for the pig industry in England for the year 2008, prior to the introduction of PCV2 vaccines, was estimated at $67.6 million per year, and approximately $113 million per year during the epidemic period (2001-2004). Currently, vaccines against PCV2 are widely used in commercial farms; however, since the vaccines do not induce sterilizing immunity, meaning it prevents symptoms of disease but not infection, the virus keeps circulating even in farms applying vaccination (Kekarainen, 2015).

The major reason this virus should be of concern to researchers is because of its potential to become zoonotic, meaning it could transfer from pigs to humans. PCV2 infects and destroys nearly every tissue and organ within its natural host, infects and induces immunosuppression in multiple species, and is capable of infecting and replicating in human cells in culture (Khayat, 2017). Because of this, further study of of how PCV2 interacts with its cellular hosts is important for the development of therapeutics to intervene with PCV2 infection. The goal of this project was to isolate the four open reading frames associated with protein products and insert them into a mammalian expression vector, with hopes of transfecting cells with the plasmids, extracting protein products, and eventually creating antibodies to these proteins.

Methods

The first step of this experiment involved plasmid DNA purification. The virus genome was given to the lab in a plasmid in DH5-alpha cells, so the genome needed to be isolated in

order to use. The QIAprep Spin Miniprep Kit protocol was used for this plasmid purification. The DNA samples were then analyzed by UV-spectrometry for concentration and purity. All samples were then standardized to 5(ng/µl) stock solution.

Next primers were developed for each of the four ORFs. The primers are as follows:

ORF1 forward: CACCATGCCCAGCAAGAAGAGTGG
ORF1 reverse: GTAATTTATTTCATATGGAAATTCAGGGCATG
ORF2 forward: CTTAGGGTTAAGTGGGGGGTCT
ORF2 reverse: CACCGCGATGACGTATCCAAGGAG
ORF3 forward: CTGATAGAATGTGGAGCTC
ORF3 reverse: CACCATGGTAACCATCCCACCACTTG
ORF4 forward: AGGACAACGGAGTGACCTCTCTAC
ORF4 reverse: CACCATGACGTGTACATTCGTCTTCC.

Once primers were designed and ready, each ORF was amplified using polymerase chain reaction (PCR). The forward and reverse primers for each ORF were used and the proofreading enzyme used was PFU, this gives fewer errors than Taq. The run cycle used was based off of the PCR Cycling Parameters for *PfuUltra* II fusion Hs DNA polymerase protocol for inserts that are less than 1 kb. Once PCR was complete, gel electrophoresis was then performed with the PCR products on a 1% agarose gel at 175V for about 2 hours. After analyzing that the gel showed the bands expected, the PCR products were the purified using the QIAquick PCR Purification Kit Protocol. These PCR products were then analyzed by UV-spectrometry for concentration and purity.

The next procedure involved cloning the PCR products into a TOPO mammalian expression vector. The protocol used was the TOPO® Cloning Procedure for Experienced Users; pcDNA3.1D/V5-His-TOPO vectors were used. The ligated expression vectors were then transformed into TOP10 chemically competent *E. coli*, and the cells were plated. Colonies were selected and harvested in LB broth and ampicillin for over 24 hour.

The quick Mini-prep protocol from the lab was then conducted, to separate plasmid DNA

from clones, and run through gel electrophoresis. Plasmids containing an insert were selected for. These plasmids were then purified using the QIAprep Spin Miniprep Kit protocol. The purified plasmids were sent out for sequencing to GeneWiz.

The sequences were received for each of the ORFs. The sequencing results for the plasmids containing ORF1, ORF2, ORF3, and ORF4 were BLASTed against the NCBI database. Each was compared to the Porcine circovirus type 2 complete genome.

Results

PCR results showed black bands of expected sizes for ORF1-4 on the agarose gel, meaning the primers designed for ORF1-ORF4 were all successful (Fig. 1)

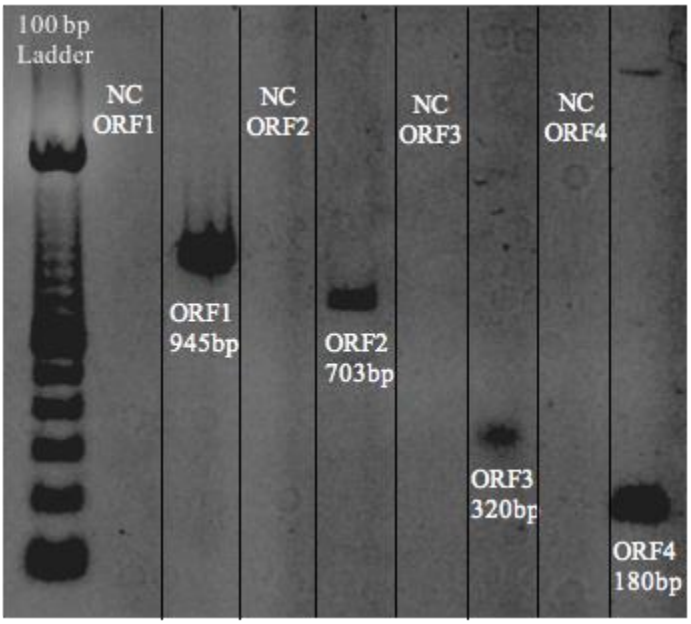
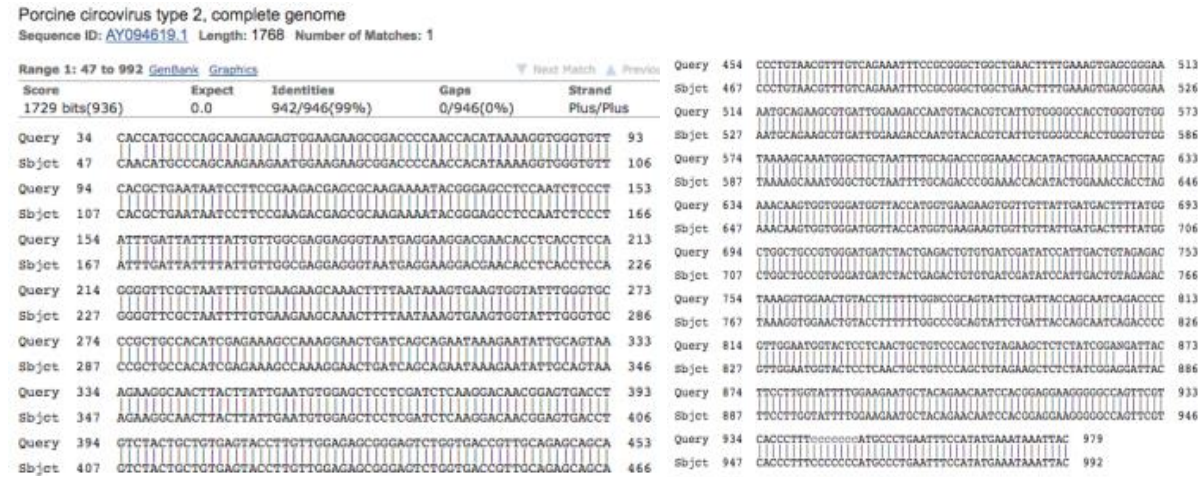


Figure 1. PCR products for ORF1, ORF2, ORF3, and ORF4, along with negative controls, on a 1% agarose gel.

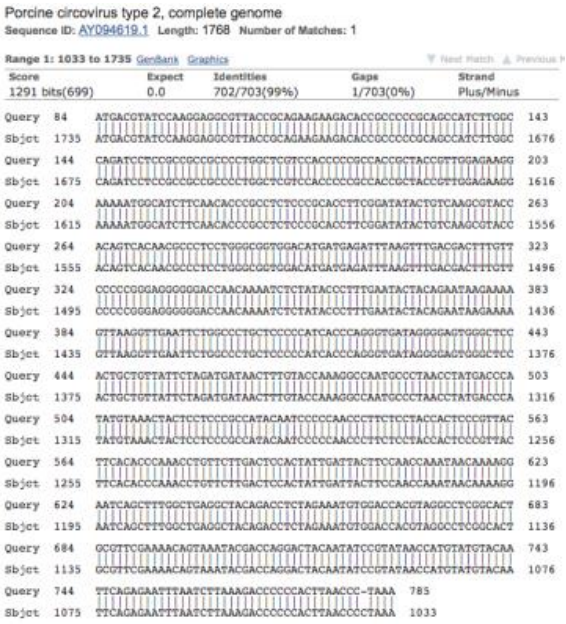
It was found that ORF1 matched the NCBI database and showed 99% of identities, with three base differences (Fig. 3a). ORF2 matched the databased and showed 99% of identities, with one base difference (Fig. 3b). ORF3 matched the database and showed 99% of identities,

with one base difference (Fig. 3c). ORF4 matched the database and showed 98% of identities, with three base differences (Fig. 3d).

a.



b.



c.

Porcine circovirus type 2, complete genome

Sequence ID: AY094619.1 Length: 1768 Number of Matches: 1

Range 1: 356 to 678 GenBank Graphics Next Match Previou

Score	Expect	Identities	Gaps	Strand
579 bits(313)	8e-169	320/323(99%)	1/323(0%)	Plus/Minus

```
Query  30   CTTCACCATGGTAACCATCCCACCACTTGTTTCTAGGTGGTTTCCAGTATGTGGTTTCCG  89
Sbjct  678  CTTCACCATGGTAACCATCCCACCACTTGTTTCTAGGTGGTTTCCAGTATGTGGTTTCCG  619
Query  90   GGTCTGCAAAATTAGCAGCCCATTTGCTTTTACCACACCCAGGTGGCCCCACAATGACGT  149
Sbjct  618  GGTCTGCAAAATTAGCAGCCCATTTGCTTTTACCACACCCAGGTGGCCCCACAATGACGT  559
Query  150  GTACATTGGTCTTCCAATCACGCTTCTGCATTTTCCCGCTCACTTTCAAAAGTTCAGCCA  209
Sbjct  558  GTACATTGGTCTTCCAATCACGCTTCTGCATTTTCCCGCTCACTTTCAAAAGTTCAGCCA  499
Query  210  GCCCGCGGAAATTTCTGACAAACGTTACAGGGTGCTGCTCTGCAACGGTCACCAGACTCC  269
Sbjct  498  GCCCGCGGAAATTTCTGACAAACGTTACAGGGTGCTGCTCTGCAACGGTCACCAGACTCC  439
Query  270  CGCTCTCCAACAAGGTACTCACAGCAGTAGACAGGTCACTCCGTTGTCCTTGAGATCGAG  329
Sbjct  438  CGCTCTCCAACAAGGTACTCACAGCAGTAGACAGGTCACTCCGTTGTCCTTGAGATCGAG  379
Query  330  GAGCTCCACATTCTATCAG-AAG  351
Sbjct  378  GAGCTCCACATTCAATAAGTAAG  356
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d.

Porcine circovirus type 2, complete genome

Sequence ID: AY094619.1 Length: 1768 Number of Matches: 1

Range 1: 389 to 569 GenBank Graphics Next Match Previou

Score	Expect	Identities	Gaps	Strand
318 bits(172)	2e-90	178/181(98%)	0/181(0%)	Plus/Minus

```
Query  33   CACCATGACGTGTACATTCGTCTTCCAATCACGCTTCTGCATTTTCCCGCTCACTTTCAA  92
Sbjct  569  CACAATGACGTGTACATTGGTCTTCCAATCACGCTTCTGCATTTTCCCGCTCACTTTCAA  510
Query  93   AAGTTCAGCCAGCCCGCGGAAATTTCTGACAAACGTTACAGGGTGCTGCTCTGCAACGGT  152
Sbjct  509  AAGTTCAGCCAGCCCGCGGAAATTTCTGACAAACGTTACAGGGTGCTGCTCTGCAACGGT  450
Query  153  CACCAGACTCCCGCTCTCCAACAAGGTACTCACAGCAGTAGAGAGGTCACTCCGTTGTCC  212
Sbjct  449  CACCAGACTCCCGCTCTCCAACAAGGTACTCACAGCAGTAGACAGGTCACTCCGTTGTCC  390
Query  213  T  213
Sbjct  389  T  389
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Figure 2. Aligned sequence for (a) ORF1, (b) ORF2, (c) ORF3, and (d) ORF4.

Discussion

The main goal of this project was to isolate the four ORFs and insert them into the pcDNA3.1D/V5-His-TOPO mammalian vector, and this was done successfully. We used the vector of choice because there are currently no commercially available antibodies for this virus that can be used in the lab. Because of this, a vector needed to be used that could be found in cells if protein products form. The V5-epitope fusion tag of the vector is helpful for this; this short peptide sequence has high-affinity antibodies against it. This allows for easy detection of recombinant proteins. Along with the V5 epitope, the 6x polyhistindine tag included in this

vector is helpful because it allows for rapid purification of protein products. Also, when using this vector, restriction enzymes do not need to be added when cloning because of the DNA Topoisomerase I that is present in the vector. This enzyme functions both as a restriction enzyme and as a ligase. It is because of this that cutting of the vector is more likely than using restriction enzymes in this scenario. All of these factors combined were important when choosing the vector to clone the ORFs into.

As mentioned, the BLAST search showed that the ORF1-4 all matched the database with either 99% or 98% of identities. The significance of the very few base differences is unknown at this current time. I will check the sequencing histogram to ensure that it is reading accordingly. These mismatches could potentially be PCR artifacts. They could also have already been present in the genome when it was first received. Further analyzation and research will need to be done before any future steps are taken.

The end goal of this project was and still is to further study the ORF's protein products, as they are recognized as the functional proteins of the virus, as well as eventually produce antibodies using purified PCV2 proteins of interest. The protocol, ideally, will first involve transfecting PK-15 cells, immortalized porcine kidney cells, with the expression vector. Then the PK-15 cells will express the transgene and produce the protein products of our genes of interest. The cells will then be lysed, and the proteins will be extracted from the cell lysate. Affinity protein purification using V5 epitope and polyhistidine-tag will purify our particular PCV2 proteins. Lastly, antibodies will be produced using rabbit or mouse immunization protocol for polyclonal antibody production or monoclonal antibody production using hybridomas. Because antibodies against this virus are not commercially available, that last step is of utter importance in order to further study the products of ORF1-4. Mechanisms of PCV2 cell cognition,

attachment, and entry are still currently being researched and not very understood. We are hoping that this research may actually help prevent PCV2 infection in humans, if such a thing occurs.

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