Acta Scientific Veterinary Sciences (ISSN: 2582-3183)

Volume 5 Issue 2 February 2023

Research Article

Expression of PCV2d Capsid Protein in *Escherichia coli* and its Evaluation for Serodiagnosis of Porcine Circovirus Infection

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DOI: 10.31080/ASVS.2022.05.0599

Received: December 16, 2022

Published: January 12, 2023

Abstract

The porcine circovirus 2 (PCV2) is one of the most economically significant swine pathogens, causing PCVAD as well as impaired reproductive efficiency, stillbirth, and weight loss in infected pig populations. The PCV2 ORF2 gene encodes capsid protein (cap protein), which has 233 amino acids (AA) in PCV2a/2b and an additional lysine residue at the C-terminus in PCV2d. In this study, we created a PCV2d capsid sequence (ORF2) for expression in *Escherichia coli*. His tag chromatography method was used to purify the protein, and western blot was used to detect protein expression. In this study, an indirect enzyme-linked immunosorbent assay (I-ELISA) based on the PCV2d ORF2 protein was developed. The best antigen, serum, and goat anti-porcine IgG conjugate dilutions were 1:1500 (3.245 µg/ml), 1:320, and 1:1600, respectively. The estimated cut-off value was 0.9885, and its analytical sensitivity was 1:2400. Except for PCV2a, PCV2b, and PCV2d antisera, the results of specific evaluation revealed that these antisera did not cross-react with any other common porcine viral pathogens, indicating that this method can be used to detect porcine circovirus infection in pig.

Keywords: PCV2d; ORF2; Capsid protein; Expression; *E coli*

Abbreviations

Nt: Nucleotide; DNA: Deoxyribonucleic Acid; TBE: Tris Borate EDTA; LB: Luria Bertani; kDa: Kilodaltons; SDS PAGE: Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis; MES: 2-(N-Morpholino) Ethane Sulfonic; HRP: Horseradish Peroxidase; ELISA: Enzyme-linked Immunosorbent Assay; Nm: Nanometer; FBS: Fetal Bovine Serum; BSA: Bovine Serum Albumin; OIE: Office International des Epizooties; ANOVA: Analysis of Variance; VLP: Virus like particle

Introduction

Porcine circovirus 2 (PCV2) is one of the most economically important swine pathogens, causing porcine circovirus associated disease (PCVAD) as well as reduced reproductive efficiency, stillbirth, and weight loss in infected pigs. PCV2 was found in domestic pig and wild boar populations across a variety of geographical regions, including China, Korea, India, United States and Europe. PCV2 infection is a major threat to India's pig industry, with outbreaks reported in Tamil Nadu, Meghalaya, and the north-eastern states [1,2].

The Porcine Circovirus Virus is a single-stranded circular DNA virus that is not enveloped and belongs to the *Circoviridae* family. The genomic DNA is 1700 nt long and is protected by a 17nm icosahedral capsid shell. Currently, two major PCV strain variants have been identified: non-pathogenic PCV1 and pathogenic PCV2. PCV1 has seven open reading frames (ORF), whereas PCV2 has four major ORF and seven additional predicted epitopes [3]. PCV2 has a faster evolutionary rate (1.2×10^{-3} substitutions/site/year) than PCV1 (1.15×10^{-5} substitutions/site/year) [4]. PCV2a and PCV2b were the most common viruses in major pig-producing countries until 2008, and most commercial vaccines are now produced against PCV2a and PCV2b.

However, a recent global epidemiological study suggests that the global pattern has shifted towards genotype PCV2d, which is now recognized as the most common genotype circulating in pig populations [5-7]. The antigenic epitope diversity of emerging PCV2d genotypes may be overlooked by current vaccines, which are primarily designed to protect against PCV2a genotypes [8].

The ORF1 and ORF2 are the most important for the virus's replication and propagation among the seven predicted open reading frames (ORF) in PCV2. By alternative splicing, the ORF1 encodes two replication proteins, Rep and Rep', both of which are required to bind to a specific octa nucleotide sequence near the stem loop structure, which is located 5'upstrem to the ORF1. In PCV2a/2b, the ORF2 gene encodes capsid protein (cap protein), which has 233 amino acids (AA), but PCV2d has an extra lysine residue at the C-terminal end. According to the structural analysis, 60 copies of capsid protein are assembled into an empty capsid particle with a diameter ranging from 17 to 30 nanometers [3]. It's also worth noting that ORF2 has a higher mutation rate than PCV2's entire genome. The capsid protein contains a number of antigenic epitopes that have been shown to interact with the host receptor and is a major immunogenic protein. The exposed loop on the surface of the capsid could be used to create immunodominant epitopes, which could be used in vaccines and diagnostic tests. The PCV2b genotype was once the most common PCV virus on the planet, but due to a recent genotypic shift, the PCV2d genotypic variant is now the most common PCV2 pathogen worldwide [9]. The goal of this study was to express and purify the capsid protein (ORF2) of porcine circovirus type 2d serotype (PCV2d) in an *Escherichia coli* host system, as well as to determine its sero-reactivity with clinical sera.

Materials and Methods

Construction of recombinant plasmid encoding PCV2d ORF2 fragment

To facilitate capsid protein gene expression, the full-length ORF2 gene of the PCV2d Indian isolate (Genebank MW125680.1) was synthesised after codon optimization and then amplified by PCR. ORF2 Forward primer 5′-GCTACCGTGGAAGACTACGGTGGC-3′ and ORF2 Reverse primer 5′-GCTTACCGGATACCTGTC-3′ were used for ORF2 gene amplification, with restriction sites *BstEII* at the 5′ end and *NotI* at the 3′ end of the sequence traversed. The amplification steps were as follows: one cycle of initial denaturation at 94 °C for 5 minutes, 35 cycles of primary amplification (denaturation at 94 °C for 1 minute, annealing at 59 °C-1 minute, and extension at 72 °C-1.5 minutes), and a final extension at 72 °C-10 minutes. The amplified PCR products were visualised on a 1.5 percent agarose gel stained with ethidium bromide and prepared in 1 TBE buffer. The gel was electrophoresed and photographed using a gel documentation system (BioRAd, USA). The PCR product encoding the PCV2d ORF2 gene was treated with *BstEII*/*NotI* before being cloned and subcloned into the pET100/D-TOPO plasmid vector (Life Technologies, USA). The precision of the PCV2d ORF2 coding sequences was confirmed using restriction enzyme digestion and DNA sequence analysis.

Expression of the recombinant ORF2 encoded cap protein in E coli host system

The transformed bacteria were plated on selective LB agar containing ampicillin 50µg/ml to form individual colonies with overnight incubation at 37°C. Individual colonies were later picked and cultured in 30 ml of LB broth supplemented with ampicillin (50µg/ml) at 37°C with constant shaking at 200 rpm overnight and cell pellet were harvested. DNA sequencing confirmed the existence of all constructs.

Purification of the recombinant protein

Single-step affinity chromatography with Ni-NTA (nickel-nitrilotriacetate) agarose (Qiagen, USA) was used to purify recombinant 27 kDa his tag fusion protein under denaturing conditions, and it was renatured as described previously [10]. Spectrophotometric analysis was used to determine the protein concentration. Filtration sterilised the protein solution, and aliquots were stored at -80°C until needed.

Expression detection using SDS PAGE

Polyacrylamide gel electrophoresis was used to separate expressed proteins using precast 4-12% Nu PAGE gels. The gels were loaded into an Invitrogen gel electrophoresis tank with 1X MES buffer. Before loading in the gel, all protein samples were mixed with 1X loading buffer (Novex) and heated to 98°C for 10 minutes. The gels were electrophoresed at a constant voltage of 200V for 30 minutes after the protein samples were loaded into the wells. A prestained protein ladder (Geneflow) was used to compare the separation of the protein samples. After electrophoresis, the gels were removed from the gel cast and either stained with Coomassie or transferred to a PVDF membrane for Western blot analysis. Protein bands were visualised by staining the electrophoresed gels with coomassie brilliant blue stain (40 percent methanol v/v, 10%

glacial acetic acid, and 0.5% coomassie brilliant blue) at room temperature for 1 hour with constant rocking. The stain was removed from the gel by incubating it overnight at room temperature with a destaining solution (40% methanol v/v, 10% glacial acetic acid).

Western blotting

Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon® Membranes, Merck, Germany) to be detected by immunoblot. Methanol was used to pre-wet the PVDF membranes, and then Western blot transfer buffer and electrophoresis were performed at 90V for 80 minutes in transfer buffer containing 20% methanol. The transfer of the prestained ladder into the PVDF membrane confirmed the complete transfer of the proteins. Membranes were blocked with HiMedia Blocking Buffer and incubated with 1:400 dilution of anti-porcine crcovirus type 2 capsid antibody (Thermofisher Scientific, Cat #PA5-34969) or PCV2d specific clinical sera, followed by three washes and incubation with polyclonal goat HRP-conjugated anti-rabbit antibody (Invitrogen, USA).

Standardization of ORF2-PCV 2d ELISA

A checkerboard titration was performed on a 96-well ELISA microplate using the traditional indirect ELISA method to optimise the concentrations of coating antigen and serum dilutions [11]. The purified ORF2-PCV2d protein was diluted twofold, from 1:1500 to 1:48000. The wells were blocked with blocking buffer (5% FBS, 10% FBS, 1% gelatin, 5% gelatin, 1% skim milk, 5% skim milk, 1% BSA, or 5% BSA) at 37 °C for 1 hour or 3 hours and then overnight at 4°C or directly overnight at 4°C. The serum samples, both positive and negative, were diluted from 1:20 to 320. IgG detection was accomplished with a goat anti-porcine IgG-horseradish peroxidase conjugate (Sigma-Aldrich, USA) that was optimised with two-fold serial dilutions (1:200 to 1:3200). All samples were measured in triplicate using an ELISA microplate spectrophotometer at 490 nm.

Validation of the assay parameters

Serum samples (n = 55) collected from various locations in India's North-Eastern region of India that were unequivocally PCV2 infected naturally and another group (n = 25) that had never been PCV2 infected based on historical data from various locations across the nation were categorised as positive and negative serum samples, respectively. The cut-off value was calculated as the average of all OD values obtained in PCV2 negative serum samples plus a 3-fold standard deviation (SD). Positive samples had OD values greater than the cut-off value were considered as true positive (TP). Serum samples were serially diluted from 1:300 to 1:38400, and end-point titration was performed to determine the ORF2-PCV2d-ELISA lower detection limit, which was defined as the maximum dilution of the sample detected just above the cut-off value [12]. PCV2-negative samples served as controls, and all samples were tested three times. According to the OIE validation recommended criteria, the diagnostic sensitivity (DSe) and specificity (DSp) were computed. The formula TP/(TP + FN), where TP stands for true positive and FN for false negative, was used to determine DSe. Similarly, DSp was calculated by the formula TN/(TN + FP), where TN: true negative; FP: false positive. Antigen cross-reactivity experiments were used to assess the antigen specificity of ORF2-PCV2d-ELISA. In the antigen cross-reactivity test, confirmed antisera to naturally infected PCV2a, PCV2b, PCV2d, Porcine reproductive and respiratory syndrome virus (PRRSV), Porcine parvovirus (PPV), Japanese Encephalitis virus (JEV), and Classical swine fever (CSF) virus were used. PCV2-negative serum samples served as controls, and each positive serum sample was tested three times.

Statistical analysis

One-way ANNOVA was used to compare the negative control group (OD) with the rest of the groups (OD).

Results and Discussion

Many positive charge arginine-rich N terminus sequences impede efficient expression and optimization of the *E. coli* expression system's sequence [13]. The full-length sequence 1-234 expresses the pcv2d capsid protein. The N-terminal 1-45 sequence encode nuclear localization signal (NLS) and 45-233 encode structural protein [3]. researchers have attempted to modify the NLS sequence or replaced with fusion tags such as maltose binding protein (MBP), glutathion-s-transferage or by adding a small ubiquitin like modifier (SUMO) motif to improve the production and efficient assembly of VLP [14]. In this study we have synthesized full-length ORF2 gene of PCV2d Indian isolate (Genebank MW125680.1) and modified by adding six consecutive histidine residues (His tag) at N terminal sequence by replacing positive charge arginine rich N terminus sequences from 9-14 residue at 5' end which might restrict efficient expression (Figure 1). The His tag also used for protein purification by immobilized metal affinity chromatography (IMAC). The 234 amino acid capsid protein (Cap) encoded by the synthetic full-length nucleotide fragment of PCV2d ORF2 is optimised for *E. coli* codon usage and expression in a pET24a (+) expression system.

The pET expression system is one of the most widely used systems in *E. coli* for cloning and *in vivo* expression of recombinant proteins due to its high translation efficiency [15]. For efficient expression of Cap protein in *E. coli*, the recombinant expression plasmid pET24-ORF2 was created, which contains the full-length codon-optimized ORF2 gene of PCV2d. SDS-PAGE revealed the production of soluble 27 kDa molecular weight in the supernatant fraction. Western blotting with anti-PCV2 swine immune serum confirmed specificity (Figure 2). Recombinant proteins overexpressed in *E. coli* tend to aggregate into insoluble inclusion bodies. The majority of expressed cap proteins were found in the supernatant fraction in Western blot analysis. The current study supported the previous experiment [15] by demonstrating that full length codon optimization outperforms partial codon optimization in terms of achieving higher levels of cap protein expression. The use of a bacterial expression system to express recombinant proteins has the advantages of being easier to maintain, scale-up, and less expensive than eukaryotic expression systems [14]. Marcekova., *et al*. (2009) synthesised a complete codon-optimized PCV2 ORF2 sequence, but it cannot be expressed in *E. coli* unless the optimized-ORF2 gene has a poly histidine tag at the N-terminus [16]. In this study, we used a fusion protein encoding the ORF2 gene that was linked to a poly histidine tag.

Figure 1: A. Alignment of nucleotide sequences from the wild-type (GenBank accession no. MW125680.1) and optimised PCV2d ORF2 genes. PCV2's full-length ORF2 (776 bp) was optimised for *E. coli* codon usage. Optimized codons are represented by "". B. Amino acid sequence alignment between the wild type and optimized sequence.

The optimal concentration of ORF2 protein as a coating antigen was 1:1500 (3.245 µg/ml), and the optimal serum and goat anti-porcine IgG conjugate dilutions were 1:320 and 1:1600, respectively. The optimal coating conditions for ORF2 protein were 1 hour at 37 °C followed by overnight storage at 4 °C. For 1 hour, 1% skim milk powder was the best blocking condition. ORF2-PCV2d-ELISA was used to test 25 PCV2-negative serum samples under optimal conditions. The OD values ranged from 0.03 to 0.96, with a mean OD value of 0.17 with the standard deviation of 0.27. The ORF2-PCV2d-ELISA method had a cut-off value of 0.9885(mean + 3 SD). The analytical sensitivity of ORF2-PCV2d-ELISA was evaluated by endpoint titration. The results showed that the analytical sensitivity of the method was 1:2400 (Figure 3). The ORF2-PCV2d-ELISA specificity evaluation results revealed that only the OD values of PCV2a, PCV-2b, and PCV-2d serum samples were above the cut-off value, while the OD values of serum from other common porcine

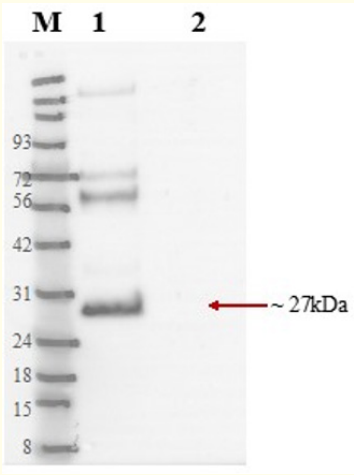

Figure 2: The expression of PCV2d_ ORF2 protein was analysed by Western blot. The blot showed serum reactivity against anti-PCV2 polyclonal sera at the anticipated molecular weights of capsid precursor proteins (~27kDa). Lane M: BLUeye Pre-Stained Protein Ladder, (Geneflow); Lane-1: Reactivity with clinical sera; Lane-2: negative control (*E coli* cell lysate).

viral pathogens (PRRSV, PPV, JEV, and CSF) were below the cut-off value (Figure 4). Estimated DSe and DSp for the developed I-ELISA is 92.7% and 84%, respectively.

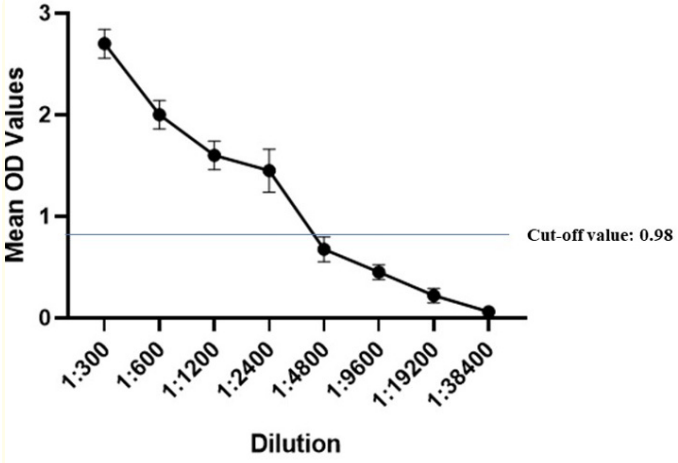

Figure 3: ORF2-PCV2d-ELISA analytical sensitivity. The ORF2-PCV2d-ELISA method was used to test PCV2d anti-serum that had been serially diluted two times (from 1:300 to 1:38400). According to the cut-off value of 0.98, the maximum dilution detected was 1:2400.

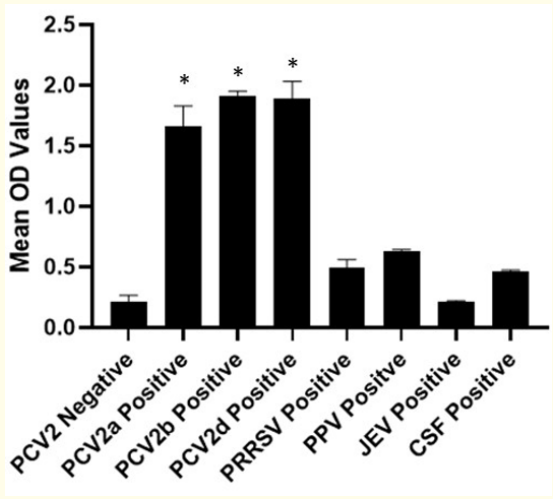

Figure 4: ORF2-PCV2d-ELISA antigenic cross-reactivity analysis. The antigenic cross-reactivity of the ORF2-PCV2d-ELISA method was investigated. Except for the PCV2a/2b/2d positive antisera, all antiserum to common pig viral pathogens had OD values below the cut-off value. *$p < 0.05$.

Conclusion

The ORF2-PCV2d-ELISA developed in this study has a high potential for early detection of porcine circovirus infection. Thus, ORF2-PCV2d ELISA can perform serological tests, giving researchers another option for monitoring PCV infection. However, the method for distinguishing PCV2 genotypes needs further investigations.

Acknowledgements

We are grateful to the Department of Biotechnology, Government of India for financial assistance (Grant No. BT/PR40363/NER/95/1636/2020).

Conflict of Interest

The authors declare that there is no conflict of interest associated with this study.

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