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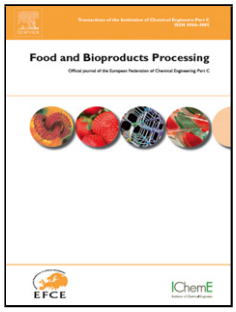

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Novel constructs and 1-step chromatography protocols for the production of Porcine Circovirus 2d (PCV2d) and Circovirus 3 (PCV3) subunit vaccine candidates

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Highlights

- Porcine Circovirus 2 vaccines are vital in preventing large-scale losses in pig farms
- There is a need to develop low-cost vaccines against the prevalent PCV2d strains
- A PCV2d vaccine candidate has been expressed at very high levels in *E. coli*
- An effective single-step chromatography protocol gives yields of over 1g / L culture
- The purified product possesses virus neutralising activity in cell based assays

Abstract

Porcine circovirus type 2 (PCV2) has been a major problem for the pig production industry worldwide for decades. While the majority of commercially available vaccines are based on the original PCV2a genotype, the current dominant genotype is PCV2d. The notable differences between genotypes could lead to incomplete cross-protection. Moreover, most current subunit PCV2 vaccines are generated from expensive insect cell culture technology. In this work, we present a new workflow for production of an updated and relatively inexpensive PCV2d vaccine candidate. After expression in fed-batch *Escherichia coli* fermentation systems with a simple one-step ion-exchange chromatography purification protocol, the yield of purified PCV2d-based antigen reached over 1 g per litre bacterial culture. Using similar procedures, we also demonstrated even higher PCV2d-based antigen yields from a chimeric PCV2d-PCV3 capsid construct, which is cleaved during fermentation to release PCV2d- and PCV3-related polypeptides. Although the PCV2d-based recombinant protein from this protocol did not form viral-like particles as analysed by size-exclusion chromatography, it could effectively induce capsid-specific and PCV2d-neutralising antibodies in immunised animals, indicating significant potential as a new vaccine candidate that can be easily manufactured at commercial scale.

Key words

Porcine circovirus 2, PCV2, E. coli., veterinary vaccine

1. Introduction

Porcine circovirus-associated disease (PCVAD) has been a massive problem for the swine industry worldwide, producing a range of symptoms such as weight loss, enlarged lymph

nodes, jaundice, respiratory distress, diarrhea and reproductive failure in infected herds (reviewed in [1-3]). Importantly, PCVAD weakens the pigs' immune systems, leading to severe secondary infections by other pathogens. Its etiological agent, porcine circovirus type 2 (PCV2), is a small non-enveloped virus, comprising only 60 copies of capsid protein monomers [1,4]. The viral genome contains two main open reading frames (ORFs). ORF1 encodes the protein 'rep' which functions in viral replication. ORF2 encodes the protein 'cap' which makes up the only structural component of the virus [1]. Despite being a DNA virus, PCV2 exhibits remarkably high evolutionary rates (1.2×10^{-3} substitutions per site per year; [5]). To date, there are six genotypes of PCV2: PCV2a, PCV2b, PCV2d (high prevalence history) and PCV2c, PCV2e, PCV2f (low prevalence history) [6,7]. Even though PCV2a-based vaccines were introduced in the early 2000s and used widely since, PCV2 is still endemic in many parts of the world and remains a major threat in swine farms.

In 2016, another related virus, designated porcine circovirus type 3 (PCV3), was discovered in pigs with porcine dermatitis and nephropathy syndrome, reproductive failure and cardiac and multi-systemic inflammation [8]. Etiological proof was demonstrated later when intranasal infection with laboratory-derived PCV3 induced clinical signs in specific-pathogen-free piglets [9]. Serological surveys demonstrated widespread presence of PCV3 in pig farms in many areas of the world (e.g. [10]. Currently, there is no commercial vaccine for PCV3.

To date, most commercial PCV2 vaccines are based on the capsid protein derived from the first genotype discovered, PCV2a [11]. Longitudinal epidemiological data indicate that PCV2a has been replaced by PCV2b and later PCV2d, currently the most prevalent strain worldwide [12]. Several studies have suggested that PCV2a-based vaccines could give

some, albeit incomplete, cross-protection to other genotypes, as pigs vaccinated by one genotype but challenged by another showed reduced viral load, but could still shed the viruses [6,13,14]. Importantly, in co-infection experiments mimicking farm conditions, a PCV2a-based vaccine was less effective than a PCV2b-based vaccine in protecting pigs against PCV2b concurrent infections with other swine pathogens [15]. Even more distant from PCV2a, PCV2d may have arisen from vaccine evasion, as it was identified as a PCV2b variant from vaccine failure cases in PCV2a-vaccinated herds [16]. Therefore, updating PCV2 vaccines to address the changing threat is crucial.

Another major concern is that commercial PCV2 vaccines can be too expensive, imposing large burdens on farmers and creating an inequitable access problem. To reduce production cost for recombinant subunit vaccines, microbial fermentation is an attractive choice. In this report, we present a new workflow for production of a PCV2d subunit vaccine candidate. With industrial-scale feasibility in mind, we generated recombinant PCV2d capsid proteins from *E. coli* expression, streamlined the tag-independent purification process to achieve a substantial yield of 1 g protein/L starting culture, and assessed the quality of the product with an industrial standard method of mass spectrometry.The resulting protein was capable of inducing neutralising antibodies in test animals, suggesting its potential as a vaccine. We also tested a chimeric protein consisting of antigens from PCV2d and PCV3 capsid proteins. Interestingly, the chimera is almost quantitatively cleaved in the linker sequence to release high quantities of PCV2d and measurable levels of PCV3. The levels of PCV2d are even higher than those obtained during expression of PCV2d on its own, demonstrating that the chimeric construct represents a novel method to produce a PCV2d vaccine candidate. Moreover, the presence of peptides derived from PCV3 capsid proteins suggests that this process can also be adapted to produce PCV3 vaccines.

2. Materials and Methods

2.1. Bacterial strains and plasmids

E. coli strains and plasmids used in this work are detailed in **Table 1.** BL21 and W3110 were used for shake flask level expression experiments and W3110 was used for fermentation experiments.

Strain/Plasmid	Description	Source
NEB Turbo	E. coli K-12 strain. F' proA B lacIq ΔlacZM15 / fhuA2 Δ(lac-proAB) glnV galK16 galE15 R(zgb-210::Tn10)TetS endA1 thi-1 Δ(hsdS-mcrB)5	NEB
DH5α	E. coli K-12 strain. F– φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1	ThermoFisher
BL21	E. coli B strain. F– ompT gal dcm lon hsdSB(rB–mB–) [malB+]K-12(λS)	ATCC
W3110	E. coli K-12 strain. F- λ- rph-1 INV(rrnD, rrnE)	ATCC
pARP25	pET23/ptac *Δ2-40* PCV2d-His6	This study
pARP30	pET23/ptac *Δ2-40* PCV2d-GSGSG-*Δ2-34, Δ195-214* PCV3-His6	This study
pARP31	pET23/ptac *Δ2-40* PCV2d-GSGSG-*Δ1-34, Δ195-214* PCV3-His6	This study
pARP34	pET23/ptac *Δ2-40 PCV2d-GGGGS-Δ1-34, Δ195-214* PCV3-His6	This study
pARP35	pET23/ptac *Δ2-40* PCV2d-EAAAK-*Δ1-34, Δ195-214* PCV3-His6	This study
pARP36	pET23/ptac *Δ2-40* PCV2d-AEAAAKALEAEAAAKA-*Δ1-34, Δ195-214* PCV3-H6	This study
pARP37	pET23/ptac *Δ2-40* PCV2d-EPEPEP-*Δ1-34, Δ195-214* PCV3-His6	This study
pARP38	pET23/ptac *Δ2-40* PCV2d - *Δ1-34, Δ195-214* PCV3-His6	This study

Table 1. Strains and plasmids used in this work.

The PCV2d ORF2 and PCV3 ORF2 coding sequences were optimised for bacterial expression based on the published amino acid sequences of *Porcine circovirus 2 isolate NPT135 capsid protein (MF314329)* and *Porcine circovirus 3 isolate PCV3-China/GD2016 (KY418606.2)*, respectively. Full length genes were cloned into a modified pET23a expression vector with ptac promoter system by restriction cloning. A construct was made containing PCV2d gene only, and another chimeric construct was made containing both PCV2d and PCV3 capsid genes joined by a short GSGSG linker region. Truncations were made to the genes by PCR amplification using 5' phosphorylated primers and direct ligation. The PCV2d N-terminal NLS region consisting of amino acids 2-40 was truncated in both single PCV2d and chimeric PCV2d-PCV3 constructs. The PCV3 putative N-terminal NLS region consisting amino acids 2-34 was truncated in both chimeric PCV2d-

PCV3 constructs, pARP30 and pARP31, using forward primer 19_ΔNLS_PCV3_F (5' P-GCAGGCACCTATTATACCAAAAAATACA 3') and reverse primer 20_Chimera_ΔNLS_PCV3_R (5' P-CATACCACTACCGCTACCTTTCGGA 3'). An additional truncation of amino acids 195-214 in the PCV3 C-terminal region of the chimeric PCV2d-PCV3 construct was made using forward primer 23_PCV3_HIS_F (5' P-CATCATCACCACCATCACTAAAC 3') and reverse primer 22_PCV3_C-term_R (5' P-GGTTTTTTCCGGAACATAAATTG 3'). Constructs pARP34 to pARP38 contain linker sequences with varying amino acid compositions and were made from pARP31 construct, also PCR amplified using 5'phosphorylated primers, using the same reverse primer 36_pARP31Linker_R (5' P-TTTCGGATTCAGAGGCG 3') and individual forward primers containing the sequence to be replaced (**Table 2**).

Plasmid	Forward primer name	Forward primer sequence
pARP34	37_Linker_overlap_F	5' P-GGTGGTGGTGGTTCTGCAGGCACCTATTATACCA 3'
PARP35	38_Linker_overlap_F	5' P-GAAGCTGCTGCTAAAGCAGGCACCTATTATACCA 3'
pARP36	39_Linker_overlap_F	5' P-GCTGAAGCTGCTGCTAAAGCTCTGGAAGCTGAAGCTGCTGCTAAAGCTGCAGGCACCTATTATACCA 3'
pARP37	40_Linker_overlap_F	5' P-GAACCGGAACCGGAACCGGCAGGCACCTATTATACCA 3'
pARP38	41_NoLinker_F	5' P-GCAGGCACCTATTATACCA 3'

Table 2. Forward primers used to generate chimeric PCV2d-PCV3 constructs with alternate linker regions.

2.2. Shake flask cell culture

Shake flask expression of recombinant proteins was initiated in 50 mL LB medium containing 100 µg/mL ampicillin by inoculation with the overnight pre-inoculant culture to an optical density (OD_{600}) of 0.05. The culture was grown at 30 °C, 200 rpm for approximately 3 hours until the OD_{600} of the culture reached 0.4-0.6. Once reached, expression was induced by addition of 100 µM IPTG. Cell samples were harvested at 3 hours and 21 hours after induction. At the specified timepoints, the OD_{600} of the culture was determined and a volume of culture equivalent to 10 OD_{600} was harvested by centrifugation at 3000 rpm for 10 minutes at 4 °C. The cell pellet was resuspended in 1 mL resuspension buffer (50mM Tris-acetate, 2.5 mM EDTA, pH 7.0) then lysed by sonication

in ice at all times, using amplitude 8.0 (Soniprep 150plus, Sanyo Gallenkamp, Loughborough, UK) for 4-6 rounds (10 s on/10 s off). Lysed suspensions were centrifuged at 14,000 rpm for 15 minutes at 4 °C and the supernatant kept as "Soluble" cell fraction, while the pellet was resuspended in a further 1 mL resuspension buffer and kept as "Insoluble" cell fraction, for analysis by SDS PAGE.

2.3. Fed-batch fermentation

A pre-inoculant culture was prepared by inoculating a single transformed W3110 *E. coli* colony into 5 mL TB media with antibiotic and grown for 6-8 hours 37 °C, 250 rpm. One mL of the pre-inoculant was transferred to a 1 L baffled flask containing 200 mL SM6Gc media with antibiotic and grown overnight at 30 °C, 250 rpm. The next day, 300 OD_{600}/L of the overnight culture were inoculated into SM6Gc medium into an Infors Multifors 1.5 L fermenter (Infors UK Ltd., Reigate, UK), to a total volume of 500 mL. To this, 100 µg/mL ampicillin and 1 mL/L PPG 2000 were added. The pH was maintained at 7.0 using 25% (v/v) ammonia solution and 25% (v/v) sulphuric acid. Dissolved oxygen tension was regulated at 40% using gas blending with 100% oxygen. The culture was grown at 30 °C until both stirrer and airflow was maximal, then the growth temperature was lowered to 25 °C. Once the culture had reached OD_{600} 38-42, a supplement of 8 mL/L 1 M $MgSO_4 \cdot 7H_2O$ was added. At OD_{600} 54-58, 5 mL/L 1.687 M NaH_2PO_4 was supplemented. At OD_{600} 60-65, 7 mL/L 1.687 M NaH_2PO_4 was supplemented, a glycerol feed (80 % (w/v) glycerol) was set at a rate of 0.025 mL/min, and protein expression was induced by adding 9 mL/L 0.0181 M IPTG. At the end of the experiment, the 500 mL culture was harvested by centrifugation in 250 mL bottles at 4000 rpm for 40 minutes at 4 °C. Cell pellets were stored at -20 °C until use. For processing, the cell pellets were resuspended in resuspension buffer (15 % w/v) and lysed using a Gaulin style homogeniser at 500 bar for

4 passages. Lysates were centrifuged at 15,000 rpm for 30 minutes at 4 °C. The supernatant containing soluble proteins was aspirated and taken forward for purification.

2.4. Cation exchange purification

Soluble cell lysates were purified by HPLC with a single step of cation exchange chromatography using a pre-packed HiTrap™ SP Sepharose HP 5 mL column (GE Healthcare, Buckinghamshire, UK), in an AKTA Pure refrigerated system, under reducing conditions using DTT unless otherwise specified. The column was loaded with the soluble cell lysate (with 0.1 mM DTT added) and washed with Binding buffer (50 mM Tris-HCl, 0.1 mM DTT, pH 7.0). Then, an elution gradient was applied in stepwise increments of 50 mM increasing salt concentration over 20 CV, using a combination of Binding buffer and Elution buffer (50 mM Tris-HCl, 1 M NaCl, 0.1 mM DTT, pH 7.0). Eluted samples were analysed by SDS PAGE and the protein content was determined by Bradford Assay.

2.5. Gel filtration chromatography

Gel filtration chromatography was carried out using Superdex™ 200 Increase 10/300 GL column (GE LifeSciences) in a refrigerated AKTA Pure system. The column was washed with 2 CV filtered and degassed water at flow rate 0.4 mL/min, then equilibrated with 2 CV Gel filtration buffer (50 mM sodium phosphate, 100 mM NaCl, pH 7.0) at flow rate 0.5 mL/min. Protein samples used had been previously purified by cation exchange without DTT. Samples with concentrations between 0.25-0.5 mg/mL (500 µL) were centrifuged at 60,000 rpm for 20 minutes, 4 °C to pellet any insoluble material prior to loading onto the column through a 1 mL capillary loop. Gel filtration buffer was run through the column (flow rate 0.5 mL/min) until no more protein peaks were observed on the UV trace. Elutions were collected in 1 mL fractions. Their molecular weights were determined using a

calibration curve created with GE Healthcare Gel Filtration Calibration HMW and LMW kits, according to manufacturer's protocol.

2.6. Sample processing for mass spectrometry

Samples were dialysed (Thermo Fisher Scientific, Slide-A- Lyzer 3.5K MWCO Dialysis Cassettes 0.1-0.5 mL) against PBS (tablets Gibco). The samples were resuspended in water, and a BCA assay (Thermo Fisher Scientific) was carried out to determine protein concentration. Finally, aliquots of 10 µg or 20 µg, respectively, were vacuum concentrated to dryness prior to further downstream processing. In-solution trypsin digestion: protein denaturation and reduction of disulfide bonds were achieved by adding 10 µL of 8 M guanidine-HCl solution (Thermo Fisher Scientific) to the PCV2d sample (10 µg or 20 µg) followed by 1 µL of 10 mM DTT (Thermo Fisher Scientific) in 50 mM ammonium bicarbonate buffer (Sigma) at pH 8.4. The solution was incubated for 30 min at 56 °C. Next, carboxymethylation of the free sulfhydryl groups in PCV2d was achieved by adding of 1 µL 55 mM iodoacetamide (Thermo Fisher Scientific) in 50 mM ammonium bicarbonate buffer at pH 8.4 followed by an incubation for 30 min at room temperature in the dark. For the results in the pilot study, we used iodoacetic acid (Sigma) instead of iodoacetamide. The reaction was diluted to 30 µl using ammonium bicarbonate buffer (pH 8.4). For the protein digestion, modified trypsin (porcine, sequencing grade from Promega) was added to the PCV2d in a ratio of 1 in 20 (trypsin:protein). The digestion was incubated at 37 °C for 12 hours. The digestion was stopped by adding trifluoroacetic acid (ROMIL, analytical grade) to a final concentration of 0.5% (v/v).

2.7. LC-MS/MS Analysis

For sample analysis, online ESI-LC-MS was used on a nanoAcquity UPLC®-system and Waters Synapt® G2-S mass spectrometer. For sample trapping, an Acquity UPLC® M-

Class Trap V/M Symmetrie® C18 (1.8 µm 75 µm x 150 mm) was applied. Higher resolution of the sample components was achieved by inserting an Acquity UPLC® M-Class column (HSS T3 1.8 µm 75 µm x 150 mm) equilibrated at 40 °C. The mobile phases was A: 0.1% (v/v) formic acid (Biosolve Chemicals) in 1 L LC-MS grade water (Greyhound) and B: 0.1% (v/v) formic acid (Biosolve Chemicals) in 1 L acetonitrile (Greyhound). Lockmass was set to m/z: 785.80 Glu-1-Fibrinopeptide B (200 nmol/µL, Waters).

For individual sample runs, the Synapt G2-S mass range was operated between 50-2000 m/z. Electrospray inlet parameters were set to a source temperature 80 °C and a capillary voltage at 2.4 kV. The resolution mode (20,000 FWHM) was recorded as a continuum MS/MS data format with a collision energy ramp from 14 to 45 eV and an intensity threshold above 2000 for triggering MS/MS measurements. MS/MS recording was switched off after 10/5 sec. Sample injection volume of 1 µL (200 fmol) was loaded for 2 min with a flow rate of 0.5 µL /min. Trapping was carried out for 5 min with a solvent mixture of 99.9% solvent A and 0.1% solvent B under a flow rate of 5 µL/min. For online sample separation, a linear gradient was chosen for 45 min ranging from 5% to 60% solvent B mixed with the required amounts of solvent A, respectively. A column wash was achieved by an initial equilibration of 97% solvent A and 3% solvent B, followed by a linear increase in solvent B to 97% (+solvent A 3%) for 5.5 min. The final equilibration period was scheduled for about 10 min with 97% solvent A and 3% solvent B. The system was operated at a constant flow rate of 0.3 µL/min at all times during the linear gradient. As reference mass Glu-1-Fibrinopeptide B (m/z: 785.8) was added.

2.8. Peptide mapping

The generated raw file data (Waters) were analysed using the BioPharmaLynx 1.3.3 and the MassLynx V4.1 software. For automated peptide mapping analysis with

BioPharmaLynx, the following parameters were considered: lock mass correction with 785.85 and 0.25 Da mass tolerance during MS^E data processing. Additionally, two missed cleavages, trypsin digestion and carbamidomethyl as fixed Cys modification. To improve sequence coverage, we allowed one methionine oxidation as a variable modification. Mass tolerance was set to 30 ppm. Mass errors observed were quite low with 3 ppm for Figure 3 and below 20 ppm for fraction 8 and 10 (see data in Figure 6). Background exclusion was based on manual signal evaluation via MassLynxV4.1 with local signal noise ratios of 1:10 threshold. This should exceed 0.01% of maximum total intensity current at all times. Intensity signals below 10 were excluded from the mapping. Protein database searches were carried out with PLGS software (Waters). The data raw file was loaded into a cap plate and rerun against the general UniProt database or general Swiss-Prot database. Search parameters were set to one missed cleavage, one oxidation as variable modification for methionine and one fixed variable carboxymethyl or carboxyamidomethyl for cysteine. Host cell proteome contamination was tested against the general Swiss-Prot *E. coli* database.

2.9. Immunofluorescence assay

PCV2d infectious virus was generated by transfection of PCV2d (strain BDH) genomic concatemers (accession number HM038017; [17]) into PCV-free PK-15 cells. Immunofluorescence assay (IFA): PCV-free PK-15 cells were plated to about 80 % confluence in a cover slide. On the next day, PCV2d (strain BDH) was incubated with the cells for 3 h at 37 °C. Following removal of the PCV2d inoculum, the cells were incubated in fresh MEM with 5% FBS at 37 °C for 72 h. Anti-PCV2d capsid antibody, produced in this study, was used as a primary antibody at the 1:1000 dilution in TBS+0.01% tween-20. Alexa488-labeled goat-anti-rabbit IgG was used as a secondary antibody, and DAPI was

used to identify cell nuclei. Fluorescence images were taken using an Olympus fluorescence microscope.

2.10. PCV2d virus neutralisation assay

Neutralisation assay based on fluorescent foci reduction was performed according to [18] with some modifications. Briefly, 50 µL of 2-fold serial dilutions of rabbit antisera (Bleed 3) in MEM were incubated with 50 µL containing 1000 PCV2d particles at 37 °C for 1 h. The undiluted pre-immunisation sera were used as a control. After incubation, the mixtures were used to inoculate fresh PK-15 cells in a 96-well plate (15,000 cells/well) at 37 °C for 3 h. After two PBS washes, 200 µL of MEM supplemented with 5% fetal bovine serum (FBS) was added to each well, and the plate was incubated at 37 °C for 48 h. PCV2d infection was later detected by immunofluorescence. After two PBS washes, cells were fixed with 80% acetone for 15 minutes at room temperatures. With PBS washes in between, rabbit-anti-PCV2d capsid antibody was used as a primary antibody and Alexa488-labeled goat-anti-rabbit IgG was used as a secondary antibody. DAPI was used to identify cell nuclei, and the numbers of infected cells (concurrence of DAPI- and Alexa488-positive nuclei) were scored on a high-content screening system (Opera Phenix). Virus neutralisation titres (VNT) were defined as the serum dilutions at which 50% inhibition of PCV2d infection occurred. Values were reported as average±SE from two independent experiments.

3. Results

3.1. Design of PCV2d and PCV2d-PCV3 capsid protein constructs and expression in E. coli

The sequence of the protein was chosen on the basis of sequence data from PCV2 strains that are prevalent in South East Asia [19], and Figure 1 shows the protein constructs chosen for this study. This PCV2d capsid protein sequence (hereafter termed PCV2d) contained deletions of the N-terminal 2-40 amino acid residues as previous studies suggested that multiple arginines in this region interfere with protein expression in bacteria [20-22]. We also expressed a chimeric construct comprising the PCV2d capsid protein sequence described above linked to a portion of PCV3 capsid protein via a short linker as shown in Figure 1. This chimeric sequence is hereafter termed PCV2d-PCV3 (amino acid sequences of both constructs are shown in Figure 6).

The sequences were cloned into the pET23a/ptac vector and expressed in *E. coli* W3110 cells grown in shake flask cultures. Expression was induced with IPTG for 3 h. Analysis of the Coomassie-stained gel shows the presence of an abundant 25 kDa band which was confirmed to be PCV2d by immunoblotting (Figure 2). The protein is found mostly in the soluble fraction. Expression of the PCV2d-PCV3 chimera results in the presence of an abundant 43 kDa band which is found primarily in the insoluble fraction, probably as inclusion bodies (Figure 2, left). However, it is notable that a significant amount of soluble PCV2d protein is present in the soluble fraction, suggesting that the chimeric construct is partially cleaved within the linker region to yield PCV2d.

3.2. Purification of PCV2d

In order to purify PCV2d in large quantities in preparation for industrial process adaptation, we cultured cells under fed-batch fermentation conditions for an extended period. Induction of PCV2d was carried out using IPTG and the cells were harvested 42 h after induction. Although the construct contains a His tag, we opted for ion exchange (IEX)

chromatography as this tag-independent method is more suitable for industrial-scale production. Once developed, the purification protocol could be transferred for a non-tag version with minimal changes. The cells were lysed, centrifuged and the supernatant was subjected to the HiTrap™ SP Sepharose FF column. The column was washed as detailed in Methods, and the PCV2d protein was eluted using a 0 - 1 M NaCl gradient.

Figure 3A shows a Coomassie-stained gel of the elution fractions. The PCV2d construct has a pI of 9.4 and at the pH used (7.0) the vast majority of the native *E. coli* proteins pass through the column without binding (not shown). The Figure shows that the later wash fractions contain relatively few proteins and the PCV2d protein elutes in large quantities late in the NaCl gradient (at an NaCl concentration of approximately 700 mM). The protein runs as a close doublet of bands on SDS-PAGE. A sample of the peak fraction was analysed by proteomic analysis. The base peak intensity chromatogram after 200 fmol injection is shown in Figure 3B. Peptide mapping of PCV2d from the resulting dataset yielded a protein sequence coverage of 95%. Most ions could be identified successfully via MS/MS fragmentation with a very low mass error +/- 3 ppm (Figure 3C). The PAGE also showed a band at 25 kDa which was established as a His-tag cleavage product (Supplementary Figure 1). Database search against the general Uniprot database resulted in a single hit for the capsid protein of the porcine circovirus 2 (accession: O56129, entry: CAPSD_PCV2) with a PLGS score of 5642.6. The mass spectrometry data here support that this single-step chromatography protocol is ideal for the production of PCV2d in large quantities and of high purity.

3.3. Production of a combination of soluble PCV2d and PCV3 using a chimeric construct

The chimeric PCV2d-PCV3 construct was designed with the aim of producing a combination of PCV2d and PCV3 for vaccine purposes, and we showed above that this construct expresses in high amounts in *E. coli*, but is insoluble when produced in shake flask cultures. However, we observed that some PCV2d, possibly cleaved from the chimera during expression, is present in the soluble fraction (Figure 2, right). These results suggest that this process, involving expression of the PCV2d-PCV3 construct, is a potential method of producing large amounts of PCV2d, and we further tested different cultivation conditions in order to assess whether yields could be enhanced.

Figure 4 shows a time course analysis of shake flask cultures grown in fermentation media of cultures expressing PCV2d alone or PCV2d-PCV3. Cells were removed after 3, 5 and 21 h and separated into soluble and insoluble fractions. These were then immunoblotted using antibodies to PCV2d. The data show that PCV2d is present in all of the samples and present in both the soluble and insoluble fractions. The data from the PCV2d-PCV3 culture show that this protein is also present at all of the timepoints, and the intact chimera is again found mostly in the insoluble fraction. However, the unexpected finding is that under these more prolonged expression conditions the vast majority of protein is cleaved and the blot shows the presence of large quantities of soluble PCV2d. The protein is present as a mix of closely grouped protein species with the most abundant being approximately the same size as PCV2d that is expressed on its own. These data indicate that the chimera is efficiently cleaved within the linker peptide to yield a series of PCV2d isoforms that differ by only a few amino acids. The combined intensities of these bands are even greater than those of the PCV2d proteins after expression of PCV2d on its own, and these results indicate that this process, involving expression of the PCV2d-PCV3 construct, is a viable method of producing particularly large amounts of PCV2d.

To confirm this point, we carried out a full fed-batch fermentation analysis similar to that used for PCV2d on its own (above). The data in Figure 5 show that the chimera is efficiently cleaved to yield soluble PCV2d throughout a full 42 h induction period; the soluble PCV2d protein is by far the most abundant protein at all time points in the PCV2d immunoblot. We then subjected the soluble fraction from the 42 h time point to ion exchange chromatography and a Coomassie-stained gel of the eluate is shown in Figure 6A. Once again, the vast majority of the native *E. coli* proteins do not bind to the column under these conditions and are found in the flow-through (FT) or wash fractions. The PCV2d elutes in a relatively pure form at a late point in the NaCl gradient. The fractions contain several lower molecular weight proteins, some of which are PCV2d or PCV3-related (see below).

To assess the overall efficiency of the expression/purification process we pooled peak fractions containing the PCV2d alone (as in Figure 3A) and determined the concentration of the protein. This gave us an overall figure of 1 g protein per litre of fed-batch culture. Similar analyses of the PCV2d derived from the PCV2d-PCV3 chimera gave a slightly higher figure, but the absence of a PCV3-specific antiserum means that we are not able to determine the concentration of PCV3 with accuracy. In order to determine whether PCV3 is indeed present, we analysed the IEX peak fractions 8 and 10 containing PCV2d-PCV3 using online ESI-LC-MS as mentioned above. The samples were injected as estimated 500 fmol/ µL and the corresponding base peak intensity chromatograms are shown in Figure 6B. The Coomassie stained protein bands of the earlier fraction 8 suggest the presence of higher background proteome compared to the later fraction 10, which appears to be more enriched in PCV2d and possibly PCV2d-PCV3 protein. Interestingly, the peptide mapping analysis for each IEX fraction indicated a nearly complete coverage of the PCV2d protein region including an intact GSGSG linker sequence. This finding was

supported by a strong MS/MS fragmentation of the individual peptides derived from the PCV2d region. However, characterisation of the PCV3 protein region was more challenging. The only identified peptide signals specific for PCV3 were present at low to very low signal-to-noise ratios (Supplementary tables 1&2- only SN>5 shown). Low intensities may correspond to signal suppression or lower quantities of PCV3 within the sample. In addition, the total sequence coverage for PCV2d-PCV3 reached similar percentages for both IEX fractions with 61.1% (fraction 8) and 57.9% (fraction 10), respectively (Figure 6C).

Based on our earlier findings and the proteomic dataset we concluded again that the chimeric PCV2d-PCV3 protein might have been accumulated in inclusion bodies after expression and could not be reliably identified in the supernatant and that it is also subject to proteolytic degradation. However, this construct could produce large amounts of high-purity PCV2d possibly supplemented with some detectable PCV3-derived peptides.

3.4. Purified PCV2d is not in the form of a virus-like particle (VLP).

Several previous studies showed that purified recombinant PCV2 can form large VLPs that resembles the intact virus in terms of overall dimensions [23-25]. We considered it important to understand whether our preparations behave in this manner and therefore subjected them to calibrated size-exclusion chromatography using a Superdex 200 column. This column separates molecules within the range of 10 kDa to 600 kDa; larger molecules elute earliest of all in the void volume. The column was calibrated with molecular weight markers and the insert in Figure 7 shows that the markers are effectively separated, with the largest protein marker (669 kDa) having a column retention volume of approximately 9.6 ml. Figure 7 also shows an immunoblot of the elution characteristics of

the PCV2d derived from cleavage of the PCV2d-PCV3 chimera; the data show that peak elution is observed with a retention volume of 17 ml; this is similar to that of the 29 kDa size marker. Very similar data were obtained with PCV2d that was expressed on its own (not shown) and these results thus show that purified PCV2d is not in the form of a VLP. Most likely, it is either monomeric or dimeric; the size exclusion results cannot distinguish between these possibilities because retention volume is not totally dependent on protein mass.

3.5. Production of antisera to PCV2d

Previous studies have suggested the importance of VLP formation for efficient PCV2 recombinant subunit vaccines. Since our protein construct does not seem to form VLP, we tested its immunogenicity in animals. Two different rabbits were immunised with purified PCV2d and the effectiveness of the antisera is shown in Figure 8A, which shows blots obtained with antisera from rabbits 1 and 2. Preliminary experiments confirmed that the pre-immune sera show no reactivity to PCV2d (not shown).

Figure 8A shows that antisera from both animals specifically recognise the PCV2d protein in a lysate of *E. coli* cells expressing PCV2d, with no evidence of cross-reactivity to other *E. coli* proteins. The antisera also detected PCV2d when diluted 1/5. The antisera furthermore detected the PCV2d-PCV3 chimera in a lysate from cells expressing this construct. A range of lower molecular mass peptides are also detected, which is clear evidence that the chimeric protein is cleaved to a series of smaller PCV2d- and PCV3-related peptides. This observation is consistent with the observation that multiple bands are apparent below the soluble PCV2d protein that is produced during fed-batch fermentation of PCV2d-PCV3 (Figure 6A). As controls we ran purified PCV3 protein and a

lysate of *E. coli* cells expressing a single chain variable antibody fragment (scFv) and no cross-reaction is observed in either case. This confirms that antisera from both rabbits specifically recognise PCV2d but not PCV3.

We further tested the antisera for the ability to recognise PCV2d capsid protein produced during PCV2d infection. PK-15 cells, which are susceptible to PCV2 infection, were mock-infected or infected with PCV2d (strain BDH). At 48 h post-infection, infected cells were stained with anti-PCV2d purified from the rabbit antisera as a primary antibody. As shown in Figure 8B, cells infected by PCV2d could be clearly labelled with anti-PCV2d antibody while those mock-infected showed a very clean background. These results demonstrate that anti-PCV2d antibody, generated by immunisation with our recombinant protein, could recognise the native structure of the PCV2d capsid protein produced during viral infection in swine cells.

3.6. The antisera can neutralise PCV2d infection in cell-based assay.

We finally addressed the key question of whether the antibodies raised in immunised animals have virus neutralising properties. A preparation of PCV2d virus was incubated with serial dilutions of antisera from each rabbit as indicated in Methods prior to infection in PK-15 cells. As a negative control, the viruses were incubated with undiluted pre-immunisation sera. PCV2d infection was detected by immunofluorescence using rabbit anti-PCV2d capsid antibody as the primary antibody and Alexa-488-labeled goat-anti-rabbit IgG as the secondary antibody. Representative figures showed significant reduction of infected cells up to 1:16 sera dilution compared to the conditions in which the virus was pre-incubated with the pre-immunisation sera (Figure 9A). Quantitative analysis on the Opera Phenix high-content screening system demonstrated the dose-dependent

neutralising activity for both rabbits, and gave the virus-neutralising (VN) titres of 28±7 for Rabbit 1 and 19±2 for Rabbit 2, while the VN titres for pre-immunisation sera were negligible (Figure 9B). The data in this section indicate that PCV2d antigen produced in our system can induce virus neutralising antibodies in test animals and suggest its potential use as a subunit vaccine.

4 Discussion

The introduction and subsequent wide adoption of PCV2a-based vaccines helped to significantly mitigate losses due to severe PCVAD symptoms in vaccinated farms. However, the emergence and perpetuation of new genotypes such as PCV2b and PCV2d could be viewed as evidence for a "leaky vaccine" situation, wherein the widespread vaccination could reduce clinical signs but could not eradicate transmission and therefore influenced evolution of heterologous viral strains [6,11]. PCV2d capsid proteins differ from those of PCV2a by 23 amino acids (out of 233/234 amino acids), including several mutations in internal epitopes and an insertion at the neutralising C-terminal epitope (reviewed in [6]). Several experiments have suggested that homologous vaccination would be more effective than heterologous vaccination for the case of PCV2. Therefore, updates in PCV2 vaccines to match currently circulating strains are highly desirable. Moreover, if the vaccines need regular updates, the development of more cost-effective and adaptable production platforms such as microbial fermentation can be highly beneficial for commercial production regimes.

The PCV2d antigen production protocols in this study offer significant potential for further development into a competitive commercial PCV2 vaccine. First, the *E. coli* fermentation platform coupled with a single-step chromatographic protocol generated particularly high yields: over 1 g purified protein per litre starting bacterial culture. This range of yield has

not been reported in previous work on microbial fermentation of PCV2 capsid proteins. For comparison, VLP production from a similar PCV2 capsid protein construct lacking the N-terminal 40 residues reported a yield of only 20 mg per litre starting culture [22]. In another report, non-tagged full-length PCV2b capsid protein produced by bacterial expression from a codon-optimized construct reached about 50 mg per litre bacterial culture [24]. A patent for preparation of a PCV2 recombinant antigen reported the antigen content produced by their process to be in the range of tens of mg per litre starting culture. Fed-batch fermentation systems are widely used in commercial *E. coli*- based production systems and there is thus every expectation that the protocol used in this study will be scalable. Second, the tag-independent 1-step purification protocol can be easily adapted to commercial-scale production. Third, the resulting protein has a level of purity that is exceptional and consistent with expectations in this field. Most importantly, the PCV2d antigen produced in this work induced strong and specific antibody production in immunised animals, and these antisera show efficient neutralising effects against the current PCV2d strain, which is a hallmark quality of successful PCV2 vaccines.

Most of the previous works have established that their candidate antigens generated by microbial fermentation formed VLPs that could subsequently induce PCV2-specific antibody responses in test animals [23-25]. It is reasonable to expect that VLPs, presenting structures that more closely resemble the viruses, could efficiently induce relevant protective antibodies. However, it has not been demonstrated empirically in test animals whether non-VLP antigens could also induce such response. In our experiments, we demonstrated via size exclusion chromatography analysis that the PCV2d antigen from these constructs did not form VLPs, agreeing with recent work in characterisation of PCV2 capsid lacking part or all of this N-terminal nuclear localisation signal [27]. Interestingly, despite lacking VLP structures, our antigen could induce PCV2d-neutralising antibodies in

test animals at reasonable VN titres. From the perspective of commercial scale production, this could become one of the important considerations as extra steps of VLP formation, purification, and characterisation will be required at additional cost during vaccine bioprocess and regulatory approvals.

One of our initial goals was to create a candidate antigen for a dual vaccine that can induce specific antibody responses to both PCV2d and PCV3. Unfortunately, efforts to generate soluble chimeric proteins by changing expression conditions, bacterial strains, or the amino acid composition of the linkers did not lead to successful solubilisation (see Methods for constructs; data not shown). Expressed at high levels, the chimeric PCV2d-PCV3 construct could be further developed by IMAC purification under denaturing conditions and refolding. Indeed, our initial purification trial could generate purified full-length chimeric PCV2d-PCV3 protein that could be used to immunise test animals. On the other hand, we showed that eluates during purification of the soluble fractions expressing this construct contained PCV3 peptides. Further optimisation, especially involving prevention of proteolytic degradation and more effective fractionations, could possibly enrich PCV3-derived peptides along with the purified soluble PCV2d antigens.

In summary, this work attempted to express and purify antigens comprising capsid proteins of the currently circulating PCV2d virus and the novel PCV3 virus. We successfully produced a PCV2d antigen in a form that is highly expressed during microbial fermentation and efficiently purified in a scalable 1-step chromatographic process. Moreover, the PCV2d-PCV3 chimera could be expressed at high levels during fermentation and the construct is efficiently cleaved to yield even higher amounts of PCV2d supplemented with PCV3-related peptides. The antigens generated from these

novel constructs demonstrated neutralising activity that indicate a high potential for use as subunit vaccines against PCV2d.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Figure 1. Design of PCV2d and PCV2d-PCV3 constructs.

The PCV2d construct was created by truncating N-terminal amino acids 2 – 40 from the full length PCV2d gene (top), while the PCV2d-PCV3 chimera construct was created by truncating amino acids 2 – 40 from PCV2d, and joining it by a short glycine-serine linker to the truncated PCV3 gene with amino acids deleted between 1 – 34 and 195-214 regions (bottom). Both were tagged with a C-terminal His6 tag for immunoblotting and purification.

Figure 2. Initial expression of PCV2d and PCV2d-PCV3 in shake flask culture. PCV2d and PCV2d-PCV3 were expressed in shake flask cultures and synthesis was induced with IPTG after 3h. The cells were pelleted, lysed and centrifuged to generate soluble and insoluble (Sol, Insol) which were analysed using Coomassie-stained SDS-PAGE (left) and anti-PCV2d immunoblot (right). The mobilities of the 2 protein constructs are indicated, and mobilities of molecular weight markers (in kDa) are shown on the left.

Figure 3. Purification of PCV2d from *E. coli* lysates using a 1-step procedure. (A) PCV2d was expressed in *E. coli* W3110 cells under fed-batch fermentation conditions. After an induction period of 42 h the cells were pelleted, lysed and centrifuged as detailed in Materials and Methods. The lysate was applied to an SP-Sepharose-FF column pre-equilibrated in 50 mM Tris-HCl, 0.1 mM DTT, pH 7.0, which was then washed with 5 column volumes of the same buffer. Elution was achieved using a gradient of 0 - 1 M NaCl in the same buffer. Samples of the wash and elution fractions were analysed using SDS-PAGE. A doublet of PCV2d proteins is indicated on the right. (B) Base peak intensity chromatogram of the PCV2d in peak fraction after an in solution digestion (DTT/IAM, trypsin for 12 hours). (C) Peptide mapping of the isolated PCV2d protein corresponding to 3B.

Figure 4. The PCV2d-PCV3 chimera is cleaved to yield high levels of soluble PCV2d during shake flask growth in fed-batch fermentation media. PCV2d and PCV2d-PCV3 were expressed in W3110 cells under shake flask conditions using fed-batch fermentation media, and synthesis was induced using IPTG. After induction periods of 3, 5 or 21 h, samples were centrifuged to pellet the cells, which were lysed and centrifuged to yield soluble and insoluble fractions (S, In). These were run on SDS-PAGE and immunoblotted using antibodies to PCV2d (see Materials and Methods). Mobilities of PCV2d and PCV2d-PCV3 are indicated.

Figure 5. Development of an extended fed-batch fermentation protocol for the production of soluble PCV2d from the PCV2d-PCV3 chimera. PCV2d-PCV3 was expressed under fed-batch fermentation conditions and samples were analysed just before induction with IPTG (0 h) and after periods of induction shown. Cells were lysed and centrifuged to generate soluble and insoluble fractions (S, In) and the fractions were analysed by immunoblotting using antisera to PCV2d.

Figure 6. Purification and analysis of soluble PCV2d derived from the PCV2d-PCV3 chimera. (A) The soluble fraction obtained from the 42 h induction time point shown in Figure 5 was applied to an SP-Sepharose-FF column, and the column was washed and eluted using the procedure described in Figure 3. Samples of the wash and NaCl elution fractions were analysed using Coomassie-stained SDS gels. (B) ESI-LC-MS chromatogram (base peak intensity) for IEX fraction 8 and 10 after an in-solution protease trypsin digestion (DTT/IAM, for 12 hours). (C) Peptide mapping of the chimera protein

PCV2d-PCV3 including the GSGSG linker at AA196-200 from fraction 8 (left) and 10 (right), respectively.

Figure 7. Purified PCV2d does not form a high molecular mass complex. Soluble PCV2d, purified after expression of the PCV2d-PCV3 chimera as detailed in Figure 6, was subjected to size exclusion chromatography using a Superdex™ 200 Increase 10/300 GL column. Elution fractions were analysed by immunoblotting with PCV2d antisera, with the PCV2d indicated and the mobilities of molecular mass markers (in kDa) also indicated on the left of the blot. The column was calibrated using a group of molecular mass standards and the peak elution point (= retention volume) for each standard is shown by arrows with the mass of the standards (in kDa) shown. The chromatogram obtained during chromatography of the standards is shown in the inset.

Figure 8. Characterisation of antisera raised against purified PCV2d. Antisera were raised against purified PCV2d by immunisation of 2 rabbits, and these antisera were tested for cross-reaction to specific proteins. (A) Two immunoblots showing reactivity (from left to right) of the sera from rabbits 1 and 2 against *E. coli* cell lysates, containing approximately 1 µg PCV2d, a 1/5 dilution of the same sample containing 0.2 µg PCV2d, 0.5 µg PCV2d-PCV3 chimera, 1/5 dilution of the same sample containing 0.1 µg PCV2d-PCV3 chimera, 0.2 µg PCV3, and 0.8 µg of a control protein, a 28 kD single-chain antibody fragment (scFv). (B) Immunofluorescence of PK-15 cells mock-infected (left) or infected with PCV2d (strain BDH; right) detected with anti-PCV2d antibody purified from the rabbi antisera. Scale bar, 50 µm.

Figure 9. Antisera can neutralise PCV2d infection in cell-based assay. (A) PK-15 cells were infected with PCV2d virus pre-treated with different dilutions of pre- or post-

immunisation sera from Rabbit 2 before imaged by immunofluorescence at 48 h post-infection. Cells infected by PCV2d showed green fluorescence. Nuclei were stained with DAPI. Figures are from a representative set of three independent experiments. Numbers below each panel (N), quantified by the high-content system detailed in Methods, indicated PCV2d-infected cells. (B) Virus neutralising titres in pre- and post-immunisation sera from two different rabbits.

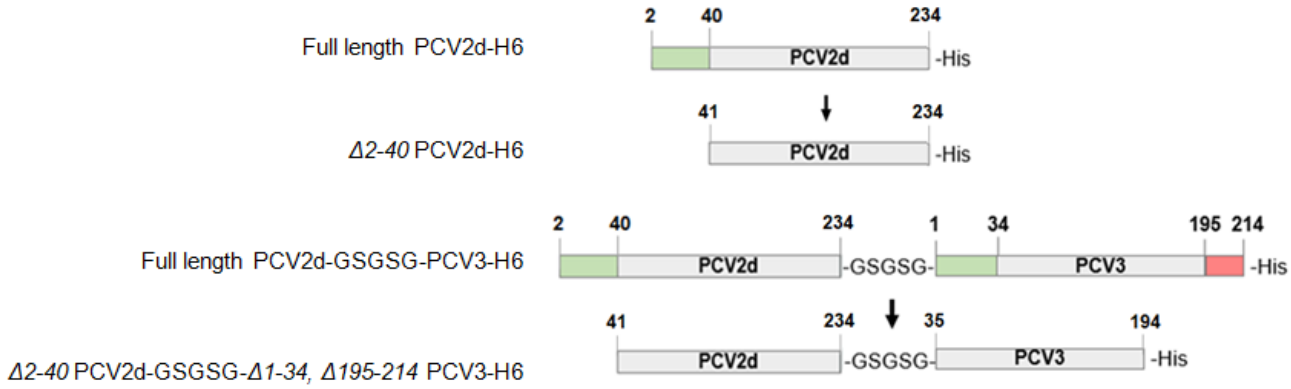


Figure 1

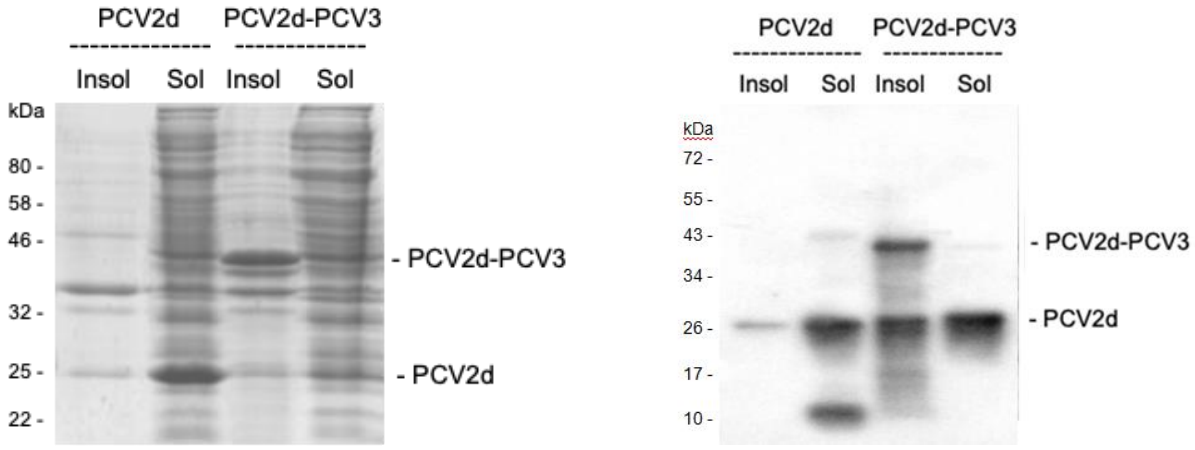
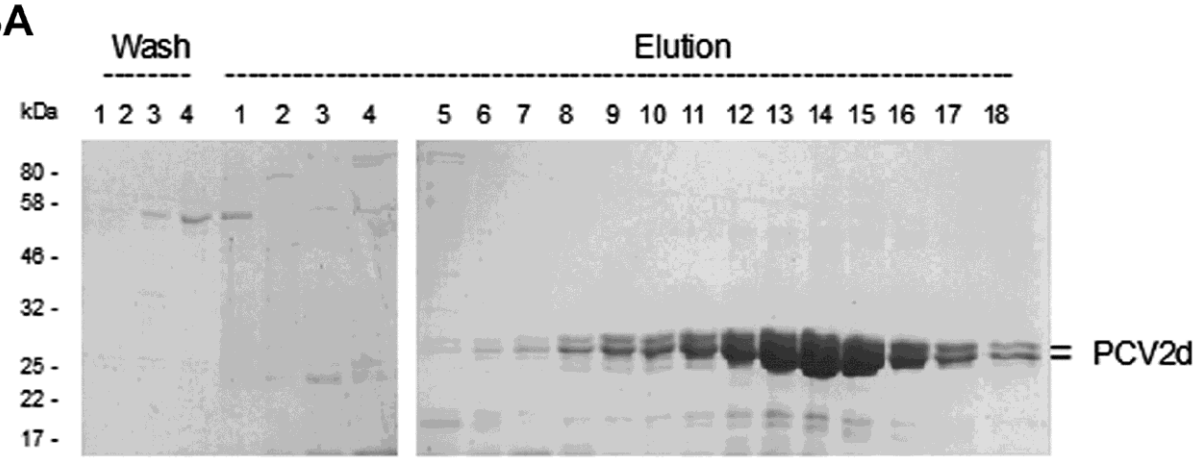
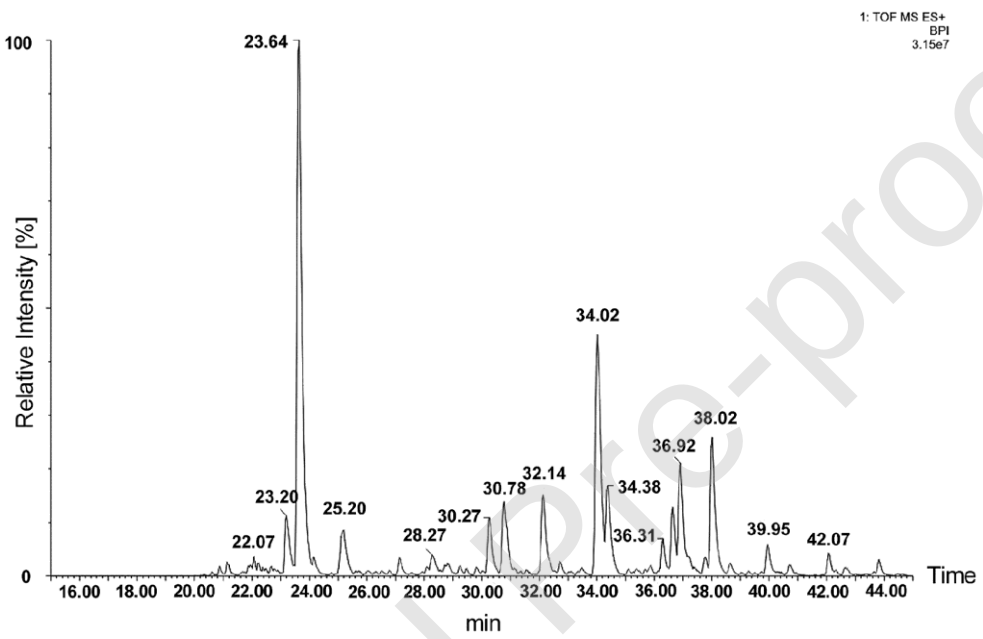


Figure 2

3A



3B



3C

PCV2d - Protein sequence coverage: 95.5%

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 51 - 100: TVPFEYYRIR KVKVEFWPCS PITQGDRGVG STAVILDDNF VTKANALTYD
101 - 150: PYVNYSSRHT ITQPFSYHSR YFTPKPVLDG TIDYFQPNNK RNQLWLRLQT
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201 - 201: H
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Figure 3

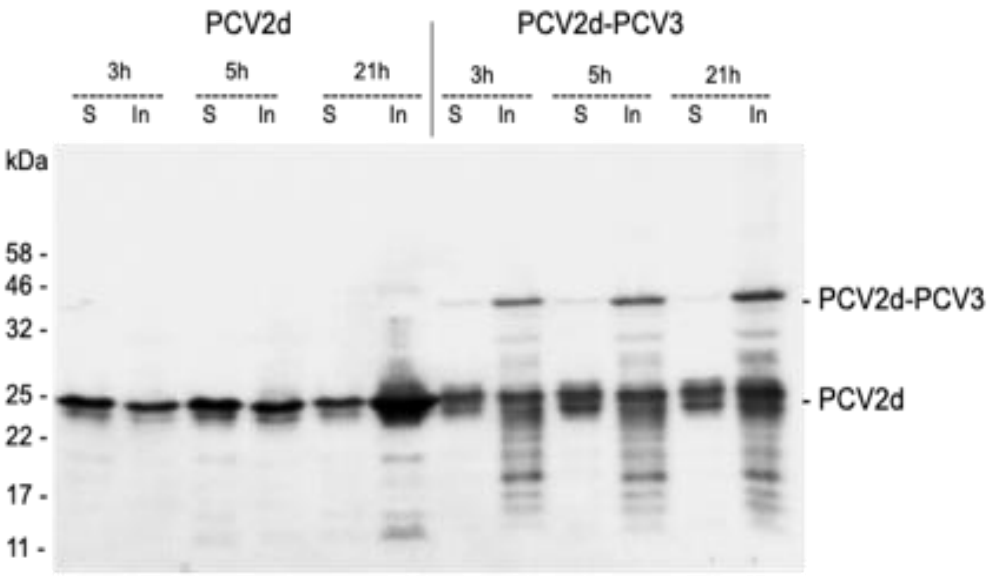


Figure 4

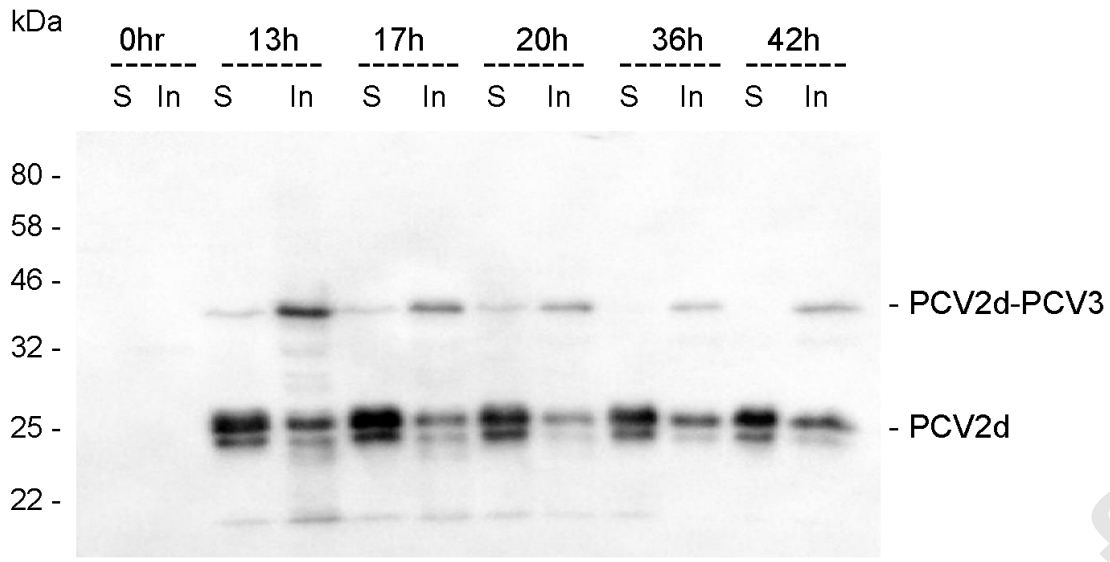
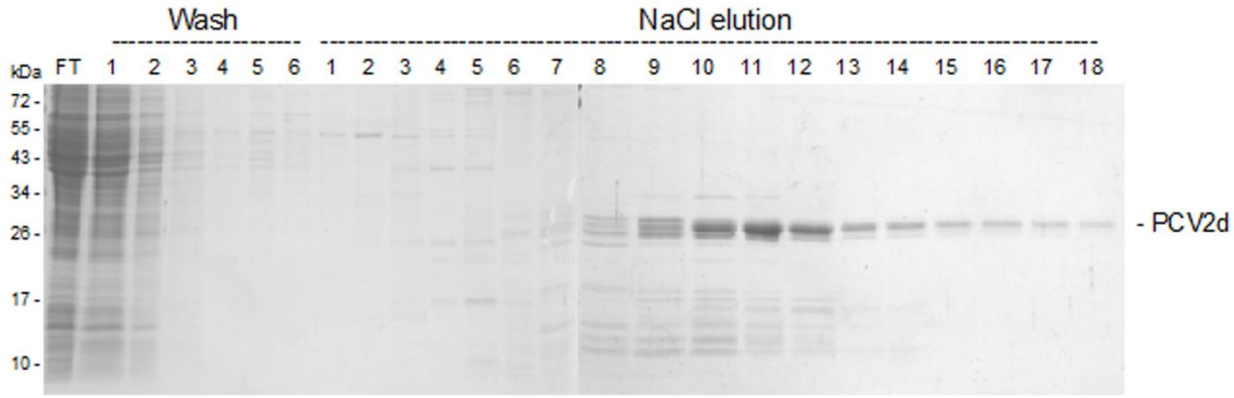


Figure 5

6A



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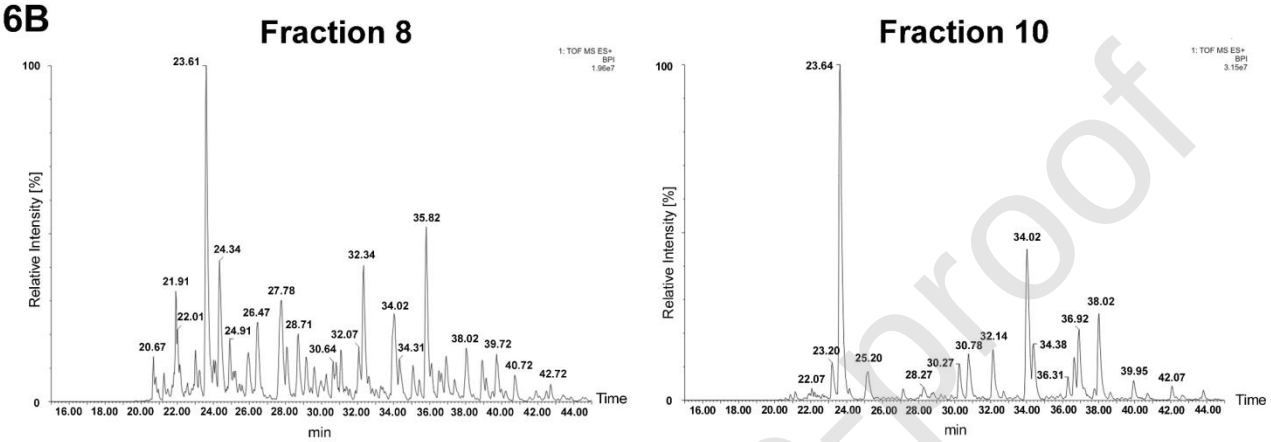

6C

Fraction E8
Sequence coverage:61.14%

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 51-100:   TVPFEYYRIR KVKVEFWPCS PITQGDRGVG STAVILDDNF VTKANALTYD
101-150:   PYVNYSSRHT ITQPFSYHSR YFTPKPVLDG TIDYFQPNNK RNQLWLRLQT
151-200:   TGNVDHVGLG TAFENSIYDQ DYNIRITMYV QFREFNLKDP PLNPKGSGSG
201-250:   MAGTYYTKKY STMNVISVGT PQNNKPWHAN HFITRLNEWE TAISFEYYKI
251-300:   LKMKVTLSPV ISPAQQTKTM FGHTAIDLDG AWTTNTWLQD DPYAESSTRK
301-350:   VMTSKKKHSR YFTPKPILAG TTSAHPGQSL FFFSRPTPWL NTYDPTVQWG
351-368:   ALLWSIYVPE KTHHHHHH
```

Fraction E10
Sequence coverage:57.88%

```
  1- 50:   MKNGIFNTRL SRTIGYTVKK TTVRTPSWNV DMMRFNINDF LPPGGGSNPL
 51-100:   TVPFEYYRIR KVKVEFWPCS PITQGDRGVG STAVILDDNF VTKANALTYD
101-150:   PYVNYSSRHT ITQPFSYHSR YFTPKPVLDG TIDYFQPNNK RNQLWLRLQT
151-200:   TGNVDHVGLG TAFENSIYDQ DYNIRITMYV QFREFNLKDP PLNPKGSGSG
201-250:   MAGTYYTKKY STMNVISVGT PQNNKPWHAN HFITRLNEWE TAISFEYYKI
251-300:   LKMKVTLSPV ISPAQQTKTM FGHTAIDLDG AWTTNTWLQD DPYAESSTRK
301-350:   VMTSKKKHSR YFTPKPILAG TTSAHPGQSL FFFSRPTPWL NTYDPTVQWG
351-368:   ALLWSIYVPE KTHHHHHH
```


Figure 6

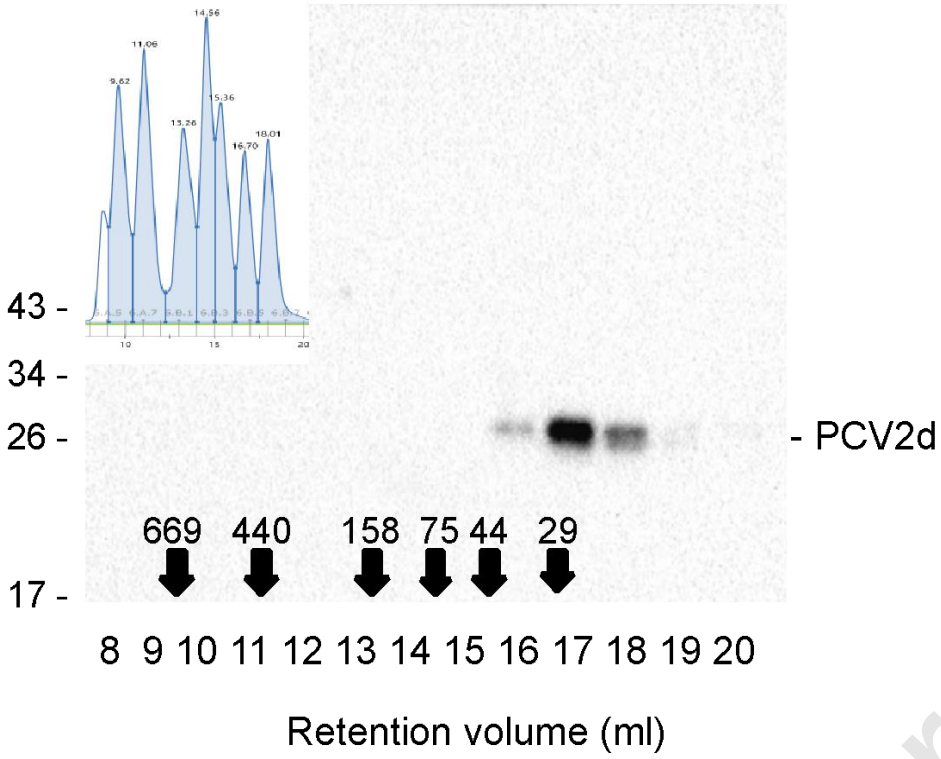

Figure 7

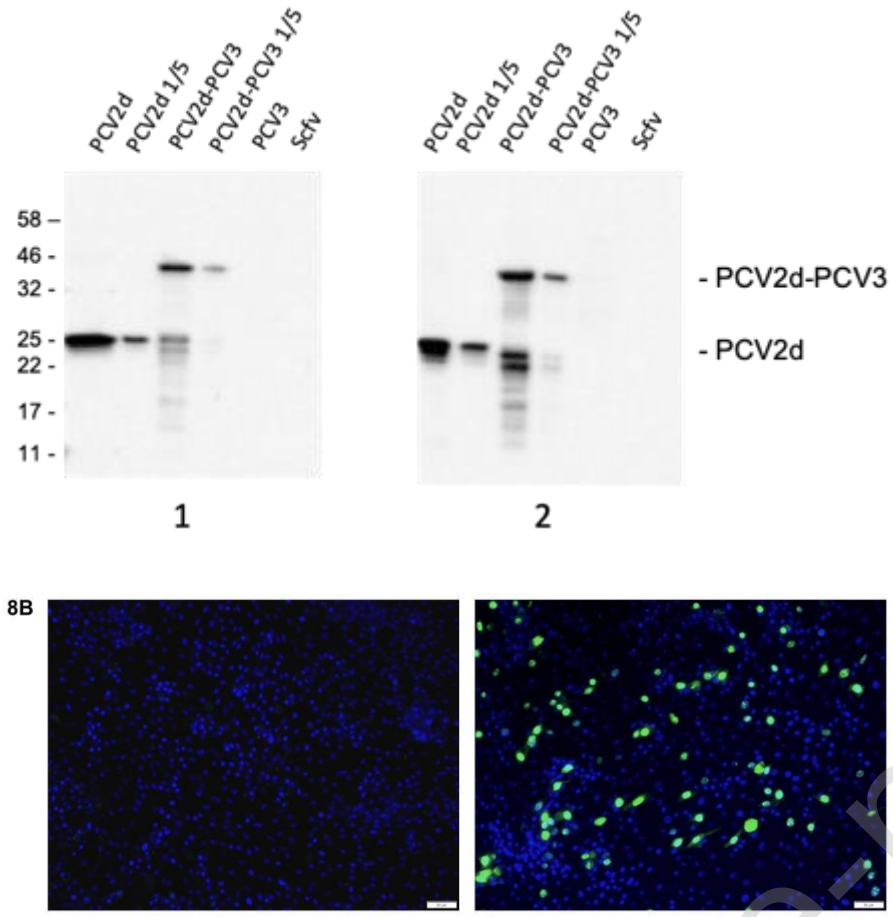

Figure 8

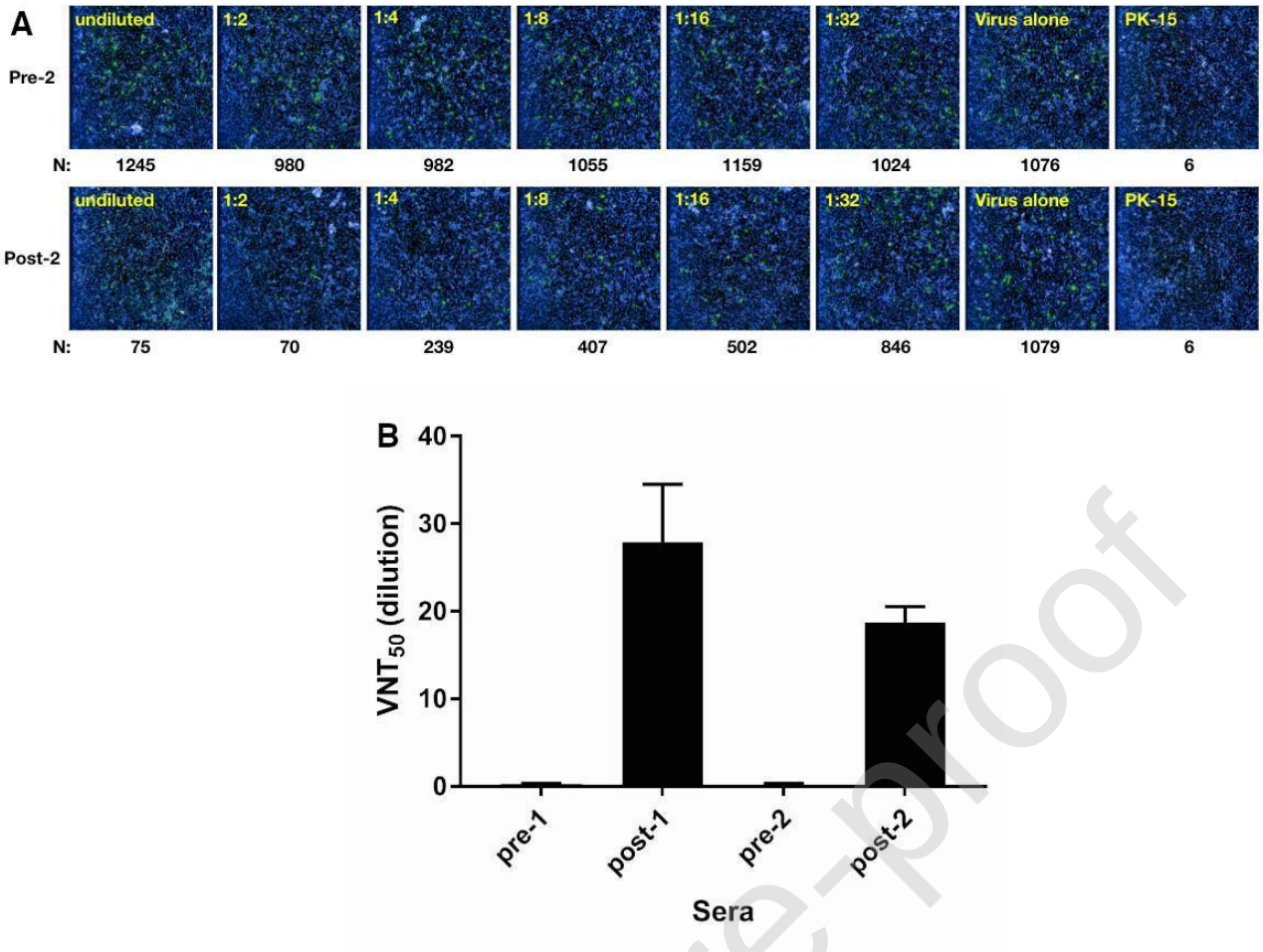

Figure 9