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Journal of Virological Methods 162 (2009) 133–141

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

# Heterologous expression of full-length capsid protein of porcine circovirus 2 in *Escherichia coli* and its potential use for detection of antibodies

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*Article history:*
Received 23 February 2009
Received in revised form 23 July 2009
Accepted 28 July 2009
Available online 5 August 2009

*Keywords:*
PCV 2
Porcine circovirus
Capsid protein
PMWS
Virus-like particles

A B S T R A C T

A capsid protein of porcine circovirus 2 (PCV 2) serves as a diagnostic antigen for the detection of PCV 2-associated disease known as a postweaning multisystemic wasting syndrome (PMWS). In this report, a bacterial expression system was developed for the expression and purification of the full-length PCV 2 capsid (Cap) protein from a codon-optimized *cap* gene. Replacement of rare arginine codons located at the 5′ end of the *cap* reading frame with codons optimal for *E. coli* was found to overcome the poor expression of the viral protein in the prokaryotic system. The Cap protein was purified to greater than 95% homogeneity by using a single cation-exchange chromatography at a yield of 10 mg per litre of bacterial culture. Despite the failure of the *E. coli*-expressed Cap protein to self-assemble into virus-like particles (VLPs), the immunization of mice with recombinant Cap yielded antibodies with the same specificity as those raised against native PCV 2 virions. In addition, the antigenic properties of the purified Cap protein were employed in a subunit-based indirect ELISA to monitor the levels of PCV 2 specific antibodies in piglets originating from a herd which was experiencing PCV 2 infection. These results pave the way for a straightforward large-scale production of the recombinant PCV 2 capsid protein and its use as a diagnostic antigen or a PCV 2 subunit vaccine.



## 1. Introduction

Porcine circoviruses (PCV) are small non-enveloped, single-stranded circular DNA viruses of the *Circoviridae* family (Allan and Ellis, 2000). Two distinct types of PCV have been described: the non-pathogenic PCV type 1 (PCV 1) (Tischer et al., 1982) and the pathogenic PCV type 2 (PCV 2) (Meehan et al., 1998; Nayar et al., 1997), which is associated with a newly emerged disease called postweaning multisystemic wasting syndrome (PMWS) (Clark, 1997). Four- to twelve-weeks old piglets which are affected with PMWS display clinical symptoms of wasting, respiratory distress, anaemia, diarrhoea, jaundice and enlarged lymph nodes (for review, see Chae, 2004). PMWS is considered to be an important porcine disease worldwide which is reported to have a serious economic impact on the global pig farming industry.

The 1.77 kB PCV 2 genome contains three functional open reading frames (ORFs) (Meehan et al., 1998). ORF1 encodes several forms of non-structural replicase proteins (Mankertz and Hillenbrand, 2001; Mankertz et al., 1998), ORF2 encodes the capsid protein (Nawagitgul et al., 2000), and ORF3 encodes a 105-amino acid protein which appears to be involved in virus-induced apoptosis of infected cells (Liu et al., 2005). The capsid protein is a unique structural protein of the viral coat (Nawagitgul et al., 2000) that is formed by 60 protein subunits in an icosahedral $T = 1$ capsid structure (Crowther et al., 2003). The N-terminus of the capsid protein is rich in basic amino acid residues and displays a nuclear localization signal (NLS) which is important for capsid assembly (Liu et al., 2001a). The capsid protein is highly immunogenic and reacts strongly with serum from PCV 2-infected pigs (Mahe et al., 2000; Fenaux et al., 2003), and thus, it is the preferred antigen in a variety of serological tests (Nawagitgul et al., 2002; Blanchard et al., 2003; Liu et al., 2004; Shang et al., 2008).

The expression of recombinant capsid protein was achieved successfully in the baculovirus system (Nawagitgul et al., 2000; Mahe et al., 2000; Blanchard et al., 2003; Fan et al., 2008), where the expressed capsid protein assembled spontaneously into virus-like particles (VLPs) (Nawagitgul et al., 2000; Liu et al., 2008). VLPs represent non-infectious structures which completely lack the DNA or RNA genome of the virus. Therefore, vaccines based on VLPs trigger an immune response to the host immune cells by mimicking native virus morphology (Ludwig and Wagner, 2007). However, the production of VLPs for vaccination in eukaryotic systems is costly for

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doi:10.1016/j.jviromet.2009.07.028

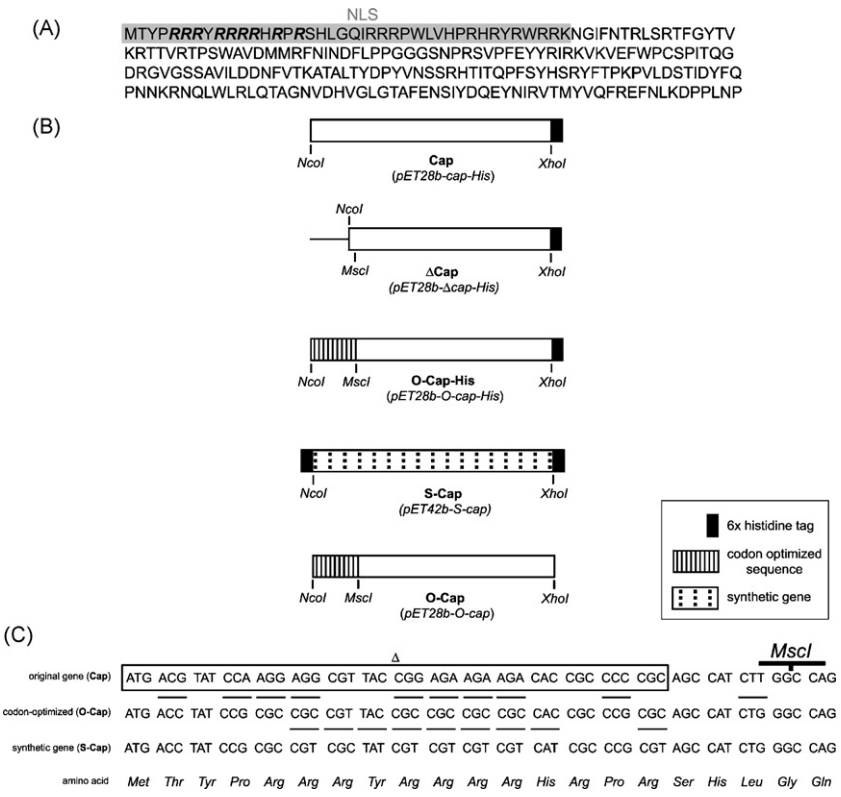


**Fig. 1.** A scheme of constructs used for PCV 2 capsid (Cap) protein expression. (A) Primary amino acid sequence of the Cap protein. The nuclear localization signal domain is in the grey box, the N-terminal arginine residues are highlighted in bold italic. (B) Schematic representation of the Cap protein variants used in this study. The designation of the protein and its expression vector is indicated. (C) Codon optimization of the 5′ end of the *cap* gene. A comparison of the original *cap* gene nucleotide sequence with the sequence of codon-optimized (*O-cap-His*) and the fully synthetic gene (*S-cap*). The modified codons are underlined. The nucleotide sequence in the box represents the deleted codons (Δ) in ΔCap-His protein. The *Msc*I restriction site in the original *cap* gene is indicated.

veterinary applications. On the other hand, heterologous protein expression in *E. coli* offers an alternative to the production of large amounts of protein. The expression of full-length capsid protein in a standard bacterial expression system, such as *E. coli* BL21 λ(DE3), has not been reported. Only certain regions of the Cap protein (Wu et al., 2008) or a fusion protein with maltose-binding protein (Liu et al., 2001b) or truncated variant of Cap lacking the NLS (Zhou et al., 2005; Trundova and Celer, 2007) have been expressed in *E. coli*.

In this study, a genetic approach which takes advantage of a codon-optimized synthetic oligonucleotide and a synthetic codon-optimized gene is described to obtain the high-level production of the full-length PCV 2 capsid protein in *E. coli* BL21 λ(DE3) cells. The purified capsid protein is used as antigen to develop an indirect ELISA for monitoring the levels of PCV 2 specific antibodies in piglets originating from a herd experiencing PCV 2 infection.

## 2. Materials and methods

### 2.1. Virus and cells

The Czech field-strain isolate of porcine circovirus type 2 (L-14181, Brno, Czech Republic) was used in this study. The virus stock was prepared from the supernatant of organ homogenate from a pig which fulfilled the diagnostic criteria for PMWS (Sorden, 2000). Samples of enlarged lymph nodes were pooled and homogenised in a fivefold volume of phosphate-buffered saline (PBS, pH 7.2). Two volumes of chloroform were added to 10 volumes of the homogenate and the mixture was shaken at 20 °C for 10 min and centrifuged at 3000 × *g* for 15 min. The supernatant was subjected to a cushion of CsCl density gradient (1.3 g/ml) and centrifuged at 60,000 × *g* for 4 h in a Beckman SW 60Ti rotor (Beckman Coulter, Fullerton, USA). The purified PCV 2 virions were resuspended in PBS and subsequently used to infect the circovirus-free PK15 cells which were maintained in a D-MEM medium (PAA Laboratories, Pasching, Austria) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Invitrogen, Carlsbad, USA) at 37 °C with 5% $CO_2$. The viral DNA was purified from PK15-infected cells using a DNAzol Genomic DNA Isolation Reagent (Molecular Research Center, Cincinnati, USA) according to manufacturer's instructions. The genomic DNA of PCV 2 isolate was sequenced by ABI Prism 3130XL analyzer (Applied Biosystems, Foster City, USA) using BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). The nucleotide sequence encoding the Cap protein was 99.9% identical with that of ORF2 of PCV 2 strain Fd4 (Genbank accession no. AY321986) (de Boisséson et al., 2004).

### 2.2. Construction of recombinant expression vectors

A 707 bp sequence encoding the Cap protein was amplified using polymerase chain reaction (PCR) with the following primers: the upstream primer 5′-CCCCATGGCGATGACGTATCCAAGGAGGC-3′ containing the *Nco*I site and the downstream primer 5′-TGTCTCGAGAGGGTTGGGGGGTC-3′ containing the *Xho*I site. The purified PCR product was cut with *Nco*I and *Xho*I (New England Biolabs, Ipswich, USA), cloned into the *pET28b* expression vector (Novagene, Merck KGaA, Darmstadt, Germany), and the vector was designated as *pET28b-cap-His* (Fig. 1B).

To generate a truncated version of the Cap protein (ΔCap-His) lacking the first N-terminal 16 amino acid residues, the *pET28b-cap-His* was used as a template for PCR with the following primers: the upstream primer 5′-AGACCATGGGCAGCC-ATCTTGGCCAGATC-3′ containing *Nco*I site and downstream primer

5′-TGTCTCGAGAGGGTTGGGGGGTC-3′ containing the *Xho*I site. The 669 bp PCR product was cut with *Nco*I and *Xho*I, cloned into the *pET28b* vector, and designated as *pET28b-Δcap-His* (Fig. 1B).

For the construction of a vector carrying the codon-optimized nucleotide sequence of the first 19 amino acid residues of the Cap protein, the *pET28b-cap-His* expression vector was cut with *Nco*I and *Msc*I. The resulting fragment was ligated with a dsDNA adaptor (sense oligonucleotide 5′-CATGACCTATCCGCGCCGCCGTTACCGCCGCCGCCGCCACCGCCCGCGCAGCCATCTGGG-3′ and anti-sense oligonucleotide 5′-CCCAGATGGCTGCGCGGGCGGTGGCGGCGGCGGCGGTAACGGCGGCGCGGATAGGT-3′) creating the *Nco*I and *Msc*I overhangs at 5′ and 3′ end of the adaptor molecule, respectively. This construct was designated as *pET28b-O-Cap-His* (Fig. 1B). Codon-optimized gene encoding the PCV 2 capsid protein was obtained from GenScript (Piscataway, USA) and the nucleotide sequence was deposited in Genbank (accession no. EU376523). The plasmid carrying the nucleotide sequence of the Cap protein was cut with *Nco*I and *Xho*I and cloned into *pET28b* expression vector. To enhance the expression of the synthetic *cap* gene in *E. coli*, a *pET42b* expression vector was modified by replacement of the nucleotide sequence between the *Nde*I and *Sac*I sites with a DNA adaptor molecule annealed from a pair of synthetic oligonucleotides (sense oligonucleotide 5′-TATGCACCACCACCACCACCACGCCATGGGAGCT-3′ and anti-sense oligonucleotide 5′-CCCATGGCGTGGTGGTGGTGGTGCA-3′) which encoded the N-terminal histidine tag of the synthetic *cap* gene. This construct was cut with *Nco*I and *Xho*I and ligated with the synthetic *cap* gene which had been cut with the same restriction enzymes to obtain the expression vector carrying S-Cap with the flanking N- and C-terminal histidine tags (*pET42b-S-Cap*) (Fig. 1B). All the constructs were confirmed by DNA sequence analysis with ABI Prism 3130XL analyzer (Applied Biosystems, Foster City, USA) using a Big Dye Terminator cycle sequencing kit.

### 2.3. Expression of recombinant proteins

Recombinant proteins were expressed in *E. coli* BL21 λ(DE3) (Novagene, Merck KGaA, Darmstadt, Germany). Cells containing the expression plasmid were grown at 37 °C in Luria-Bertani (LB) medium supplemented with 60 μg/ml kanamycin to the optical density of 0.6 at 600 nm and then isopropyl β-D-thiogalactopyranoside (IPTG) (Alexis Corporation, Lausen, Switzerland) was added to a final concentration of 1 mM. After 4 h of growth, cells were harvested by centrifugation (4000 × *g* for 20 min) and resuspended in 100 mM Tris–HCl (pH 8.0) containing 1 mM EDTA. The expression in *E. coli* BL21-CodonPlus(DE3)-RIPL strain (Stratagene, Agilent Technologies, Santa Clara, USA) was done under the same conditions, except for antibiotics concentrations: kanamycin (30 μg/ml), chloramphenicol (34 μg/ml) and spectinomycin (75 μg/ml). This strain contains extra copies of the argU, ileY, proL, and leuW genes for rare tRNAs, which can rescue protein production restricted by either AGG/AGA or CCC codons. The expression of desired proteins was documented by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

### 2.4. Protein purification

Bacterial pellet was washed and resuspended in sonication buffer (50 mM MES, pH 5.8, 50 mM NaCl) containing 1 mM phenylmethylsulfonyl fluoride. The cells were disrupted by sonication (45 W, Misonix sonicator 3000, Misonix, Farmingdale, USA) on ice and the homogenate was centrifuged at 15,000 × *g* at 4 °C for 30 min. Solid urea was added to a final concentration of 8 M, and mixed gently for 30 min. After centrifugation at 15,000 × *g* for 10 min at 4 °C, the supernatant was loaded to a UNOsphere-S cation-exchange column (Bio-rad, Hercules, USA) which was connected to an AKTA prime chromatography system (GE Healthcare, Chalfont St. Giles, United Kingdom). After washing with 10 column volumes of sonication buffer, the Cap protein was eluted with a continuous gradient of NaCl (0–1 M) in a buffer containing 50 mM MES, pH 5.8 and 8 M urea. Pooled fractions of purified Cap protein were concentrated by using Centricons (Amicon MW 10000 cut-off, Millipore, Billerica, USA), and stored at −20 °C for further use. Total protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, USA) using bovine serum albumin as a standard.

### 2.5. SDS-PAGE and Western blotting

Proteins were separated by SDS-PAGE using 12% polyacrylamide gels and either stained by Coomassie brilliant blue R-250 or transferred to a nitrocellulose membrane (Pall Corporation, New York, USA) in a transfer buffer (20 mM Tris–HCl, pH 8.3, 190 mM glycine, 0.1% SDS, 20% methanol) using a TE 70XP Semi-Dry Transfer Unit (Hoefer, Holliston, USA) at 1 V/cm$^2$ for 1 h. The membranes were blocked with 5% non-fat milk in PBS-T (phosphate-buffered saline containing 0.05% Tween-20), incubated with anti-His monoclonal antibody (1:5000) (Sigma, St. Louis, USA) followed with a peroxidase-conjugated anti-mouse antibody. The signal was developed using an enhanced chemiluminiscence system (GE Healthcare, Chalfont St. Giles, United Kingdom).

### 2.6. Preparation and characterization of polyclonal antibody

The experimental work with mice was conducted according to the certificate of animal welfare authority commission of the Ministry of Agriculture of the Czech Republic No. MZe 926/08 in accordance with current Czech legislation on animal welfare. A total of 15 Balb/c mice (8 weeks of age) were used in the experiment. A group of 5 mice was bled before the immunization to obtain pre-immune serum. A group of 10 mice received intramuscular injection with recombinant S-Cap (25 μg protein per mouse) mixed with a complete Freund's adjuvant (Sigma, St. Louis, USA). Three weeks later, the mice were boosted intramuscularly with the same dose of recombinant Cap protein mixed with incomplete Freund's adjuvant (Sigma, St. Louis, USA). The mice were euthanised and bled 24 days after the last injection prior to the sera were screened for the presence of the Cap-specific antibodies by using an immunoperoxidase monolayer assay (IPMA). For IPMA, confluent monolayers of PK15 cells were mixed with a reference PCV 2 isolate (Stoon-1010, after 36 passages) and incubated at 37 °C for 48 h prior to fixation with 4% paraformaldehyde. The fixed cells were then incubated with mice sera (1:50 dilution in PBS-T buffer) at 37 °C for 1 h, followed by incubation with a peroxidase-conjugated goat anti-mouse secondary antibody (1:500 dilution in PBS-T buffer) and the peroxidase activity was detected with 4-chloro-1-naphthol (Bio-Rad, Hercules, USA) as a substrate.

### 2.7. Electron microscopy

*E. coli* cells were washed twice in 50 mM phosphate buffer (pH 7.4) and fixed in a solution containing 2% glutaraldehyde in 50 mM phosphate buffer (pH 7.4) for 2 h. After three washes, the cells were post-fixed in buffered 1% $OsO_4$ (50 mM phosphate buffer, pH 7.4) for 1 h, washed three times in 50 mM phosphate buffer (pH 7.4), dehydrated in ethanol series, and then transferred into absolute acetone and embedded in Vestopal W resin (Sigma, Hercules, USA). Ultrathin sections were cut with glass knife using a LKB Ultratome 1 (LKB, Bromma, Sweden) and mounted on Formvar-coated copper grids. The grids were stained in saturated aqueous uranyl acetate followed by lead citrate. Samples were examined under Philips CM100 electron microscope (FEI, Eidhoven, The Netherlands) at

80 kV. Images were recorded by using a MegaViewII slow scan camera controlled by AnalySis 3.2 software (Olympus Soft Imaging Solutions, Münster, Germany).

### 2.8. Detection of antibodies against PCV 2 by ELISA

PCV 2 antibodies were determined with an indirect enzyme-linked immunosorbent assay (ELISA) using the purified S-Cap and ΔCap-His proteins as coating antigens (Cap-ELISA). The 96-well ELISA plates (Nunc, Thermo Fisher Scientific, Waltham, USA) were coated with 50 μl of 100 mM sodium carbonate buffer (pH 8.9) containing 10 μg/ml of the proteins and incubated overnight at 4 °C. The plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T) and blocked with a blocking buffer (PBS containing 1% bovine serum albumin) at 37 °C for 1 h. After washing, 100 μl of each serum sample (diluted 1:100) was added into each well and tested in quadruplicate: two wells for a negative control antigen Glutathione S-transferase (Sigma) and two parallel wells for the antigens. The plates were washed with PBS-T and 50 μl of blocking buffer containing a peroxidase-conjugated goat anti-swine antibody (Bethyl Laboratories, Montgomery, USA) was added into each well and incubated for 1 h. Finally, the plates were washed with PBS-T three times and the colour reaction was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate. The optical density at 450 nm ($OD_{450nm}$) was read using a microplate reader. The final $OD_{450nm}$ value was calculated by subtracting the mean value of $OD_{450nm}$ of wells containing a negative antigen from that of the parallel wells containing the capsid proteins. The cut-off value was determined by using specific pathogen free (SPF) pig's serum samples diluted at 1:40 which were negative for anti-PCV 2 antibodies as determined by IPMA. The mean $OD_{450nm}$ value was 0.098 with a standard deviation (S.D.) of 0.035 and the final cut-off value was calculated by adding the mean $OD_{450nm}$ value + 3 S.D. to the value of 0.200. Pig serum with a high titre of PCV 2 antibodies 1:5120, as determined by IPMA, was used as a positive control (kindly provided by Annette Mankertz from Robert-Koch Institut, Berlin, Germany). The SPF pig sera were negative for PCV 2 and used as negative controls in each plate.

### 2.9. Time course analysis of PCV 2 infection in piglets

A limited serological and genomic load survey of specific PCV 2 antibodies and equivalent PCV 2 genome copy numbers, respectively, was carried out on pig sera collected at 4, 8, 12, 16 and 20 weeks of age. 22 selected piglets were selected from a pig herd experiencing PCV 2 infection, marked with tags and monitored during their nursery and fattening period. At the regular intervals, a minimum of 2 ml of blood was collected from each piglet and the sera were stored at −20 °C for further use.

### 2.10. Isolation and quantitative determination of PCV 2 DNA

Total genomic DNA was extracted from a 200 μl volume of each serum sample using a NucleoSpin Blood isolation kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Quantification of PCV 2 DNA levels was performed using a real-time PCR by Roche Light Cycler 480 (Roche, Basel, Switzerland) in a TaqMan format as previously described (Brunborg et al., 2004). In short, the TaqMan probe was labelled with 5′-FAM and 3′-BHQ1 fluorophores (Generi Biotech, Hradec Kralove, Czech Republic) and the primers were designed to amplify a 100 bp DNA segment within the nucleotide sequence of the PCV 2 *cap* gene. The absolute quantification of PCV 2 DNA was carried out using calibration curves generated by means of external standard DNA obtained by cloning the PCV 2 *cap* gene into a *pCR 2.1* vector (Invitrogen, Carlsbad, USA). Standard curves for PCV 2 DNA quantification were generated using tenfold dilution of the linearised plasmids in the range of 8 $\log_{10}$.

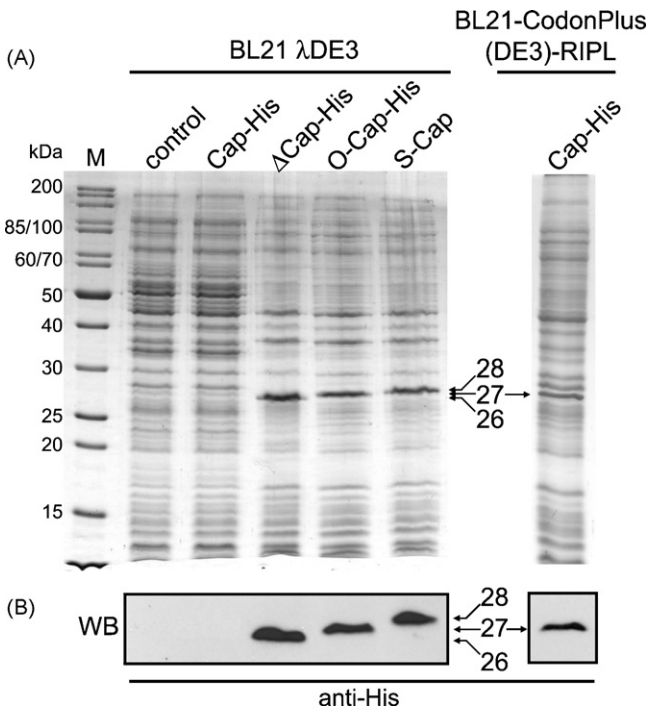


**Fig. 2.** The expression of various Cap proteins in *E. coli*. (A) SDS-PAGE analysis of cellular proteins obtained from IPTG-induced *E. coli* cells. M, molecular weight markers (in kDa); control, control cells (no IPTG); Cap-His, expression of the Cap-His protein; ΔCap-His, expression of the ΔCap-His protein; O-Cap-His, expression of the O-Cap-His protein; S-Cap, expression of the S-Cap protein. The expression of the Cap-His protein in *E. coli* BL21-CodonPlus(DE3)-RIPL is indicated on the right. (B) Western blot analysis of total cellular proteins obtained from IPTG-induced *E. coli* cells by using anti-His antibody. The blot corresponds to lanes 2–6 from (A). The arrows represent the location of the ΔCap-His (26 kDa), O-Cap-His (27 kDa), S-Cap (28 kDa), and Cap-His proteins, respectively.

### 2.11. Statistical analyses

Statistical analyses were carried out using the one-way analysis of variance (ANOVA) test running in SPSS software (Base 14.0, SPSS, Chicago, USA). Values of $p < 0.05$ were considered significant.

## 3. Results and discussion

### 3.1. Expression of recombinant capsid proteins

The open reading frame encoding Cap protein was amplified using PCR from genomic DNA of PCV 2 (L14181 isolate) and the 707 bp long DNA fragment was cloned into *pET28b* expression vector (Fig. 1B). The expression was documented in crude cell lysate by SDS-PAGE and Western blotting. As demonstrated in Fig. 2, no expression of Cap-His was detected in Coomasie-stained gels and by Western blot using anti-His antibody.

Frequently, an insufficient production of heterologous proteins in *E. coli* is due to the presence of codons that are used rarely in bacterial proteosynthesis (Zahn, 1996). Forced high-level expression of genes with rare codons may lead to the depletion of the endogenous pools of the corresponding tRNAs, resulting in the abortion of the translation process and the degradation of the mRNA (McNulty et al., 2003). Examination of the nucleotide sequence of the *cap* gene revealed a cluster of rare codons within the first 16 codons of the gene (Fig. 1C). The presence of such rare codon tandems near the 5′ end of a coding sequence has been reported to cause ribosomal

frameshifts and codon skipping with a strong inhibitory effect on protein translation (Gurvich et al., 2005).

In order to eliminate the cluster of rare codons from the 5′ end of the *cap* gene, the expression vector encoding a truncated variant of Cap protein (ΔCap-His), which was lacking the first 16 amino acid residues, was constructed (Fig. 1B). Expression of the truncated gene in IPTG-induced *E. coli* BL21 λ(DE3) cells resulted in the production of a protein with an apparent molecular weight of 26 kDa (Fig. 2A) which corresponded to the ΔCap-His protein. This suggested that the removal of the first 16 codons from the 5′ end of the *cap* gene allowed the production of Cap protein in *E. coli*. In previous report, the expression of the truncated variant of Cap protein lacking the nuclear localization signal (NLS) has been reported (Zhou et al., 2005; Trundova and Celer, 2007). The NLS consists of 41 amino acid residues at the N-terminus of Cap and the NLS-defective constructs have been expressed also as fusion proteins with Glutathione S-transferase (Zhou et al., 2005) or maltose-binding protein (Liu et al., 2001b). Based on these results, the authors concluded that the expression of the capsid protein in *E. coli* was inhibited by the nucleotide sequence encoding the NLS domain. However, the expression of the truncated Cap protein, which lacks the first 16 amino acids, was achieved which suggested that the production of the full-length capsid protein in *E. coli* was hindered only by the occurrence of rare arginine codons near the 5′ end of the *cap* gene.

The heterologous expression of the entire *cap* gene was examined also in *E. coli* BL21-CodonPlus(DE3)-RIPL cells. This strain contains extra copies of the argU, ileY, proL, and leuW genes for rare tRNAs, which rescue protein production restricted by either AGG/AGA or CCC codons. The expression of the *cap* gene in IPTG-induced *E. coli* BL21-CodonPlus(DE3)-RIPL yielded the production of a protein with an apparent molecular weight of 27 kDa which corresponded to the full-length Cap protein (Fig. 2A). This indicated that the expression of rare aminoacyl-tRNAs allowed the production of the full-length Cap protein. However, the use of *E. coli* BL21-CodonPlus(DE3)-RIPL for a high-yield production of recombinant proteins is inconvenient as the expression system depends on using a combination of three different antibiotics: one for the maintenance of the expression vector, and the other for the maintenance of plasmids carrying tRNA genes. Moreover, due to the presence of high amounts of antibiotics the growth rate of bacterial cells is reduced and expensive.

A promising approach to maximise heterologous production of proteins in *E. coli* is codon optimization strategy which makes codon usage in the gene of interest to match the available tRNA pool within the cells (Jana and Deb, 2005; Peti and Page, 2007; Burgess-Brown et al., 2008). To facilitate the *cap* gene expression in *E. coli* BL21 λ(DE3), the sequence of the first 19 codons was optimized as described in Fig. 1C. Ten codons, rare in *E. coli*, were replaced by the most frequent ones, including six arginine, two proline, and single leucine and threonine codon substitutions. The codon-optimized sequence was introduced into *pET28b-cap-His* using *Nco*I and *Msc*I restriction sites to obtain *pET28b-O-cap-His* (Fig. 1B). The expression of the *O-cap-His* gene in *E. coli* BL21 λ(DE3) cells yielded the production of a protein with a molecular weight of 27 kDa (Fig. 2A). The corresponding band was recognised by the anti-His antibody which confirmed the presence of the O-Cap-His protein (Fig. 2B). These data indicated that the replacement of the first 19 codons of the *cap* gene with a codon-optimized sequence enabled the production of Cap protein in a conventional prokaryotic expression system, such as *E. coli* BL21 λ(DE3).

Different bioinformatics tools are available for codon optimization which considers many factors, such as RNA secondary structure, GC content, repetitive and rare codons (Grote et al., 2005). These approaches were used to design a synthetic *cap* gene with nucleotide sequence optimized for prokaryotic expression in *E. coli* (Genbank accession no. EU376523). The synthetic gene with introduced *NcoI* and *XhoI* restriction sites was cloned into *pET28b* vector carrying the C-terminal polyhistidine tag and the expression of this construct was analyzed by SDS-PAGE. However, no protein product was detected in Coomasie-stained gel (data not shown). To force the expression of the capsid protein from the synthetic gene, additional codons encoding the polyhistidine tag were added at the 5′ end of the gene. The expression of this construct (*pET42b-S-Cap*) in *E. coli* BL21 λ(DE3) yielded the S-Cap protein with a molecular mass of 28 kDa, which was a slightly higher (+1 kDa) than the O-Cap-His protein due to the presence of the additional polyhistidine tag at the N-terminus of the protein (Fig. 2A). The levels of the S-Cap protein expression were comparable to those of the O-Cap-His protein which indicated that codon optimization of the entire sequence of the *cap* gene did not enhance significantly the heterologous expression of the capsid protein in *E. coli*.

### 3.2. Purification of Cap proteins

The analysis of protein distribution in *E. coli* cells revealed that all the Cap proteins were recovered in the soluble (cytoplasmic) fraction of the IPTG-induced cell lysate. However, initial attempts to purify native Cap proteins gave unsatisfactory results. Using standard chromatographic procedures, no significant binding of Cap proteins was detected while loading the material on either cation-exchange or nickel immobilised affinity columns in native buffer conditions (data not shown). To enable purification of Cap proteins, denaturating conditions in the presence of 8 M urea were utilised. The cytosolic extract of the cell lysates was supplemented with solid urea to a final concentration of 8 M and the mixture was loaded onto a UnoSphere-S cation-exchange column at pH 5.8. A typical elution profile of protein fractions from the UnoSphere-S column is shown in Fig. 3A. Fractions 1–14 contained large amounts of unrelated bacterial proteins, while the later fractions, eluting after 38 min, were enriched with Cap proteins (Fig. 3B). The identity of the Cap proteins was confirmed also by Western blotting with anti-His antibody (Fig. 3C). However, some apparent differences between the S-Cap protein and the remaining capsid proteins were observed during purification procedures. While the S-Cap protein was recovered in a high purity in the distinct peak eluting at 40 min (Fig. 3B, fraction 17), both the O-Cap-His and Cap-His proteins were eluted from the column in a shorter time (about 38–39 min). Moreover, these fractions were enriched partially with a protein with a molecular weight of about 30 kDa and some low molecular weight proteins below 15 kDa (data not shown).

The original aim of using the polyhistidine tag was to isolate the Cap proteins on a metal affinity chromatography column. However, the presence of two polyhistidine tags within the S-Cap protein resulted in the increase of the basicity of the protein, which enabled simple purification of the protein by a single step cation-exchange chromatography. The next purification steps did not increase the purity of proteins (data not shown) and these steps were omitted in the purification protocol. The purity of the S-Cap protein was greater than 95% after the cation-exchange chromatography and approximately 10 mg of purified protein was obtained from 1 l of bacterial cell culture. These data suggested that the yield of the S-Cap protein is much higher than the yield of the O-Cap-His and Cap-His proteins in terms of purity, time and costs for large-scale production of the recombinant PCV 2 capsid protein.

### 3.3. Immunogenicity of S-Cap protein

To examine the immunogenicity of the S-Cap protein, mice were immunized with the purified protein and the collected sera were analyzed for the presence of Cap-specific antibodies. Western blot analyses revealed that a single 28-kDa band in the bacterial lysate

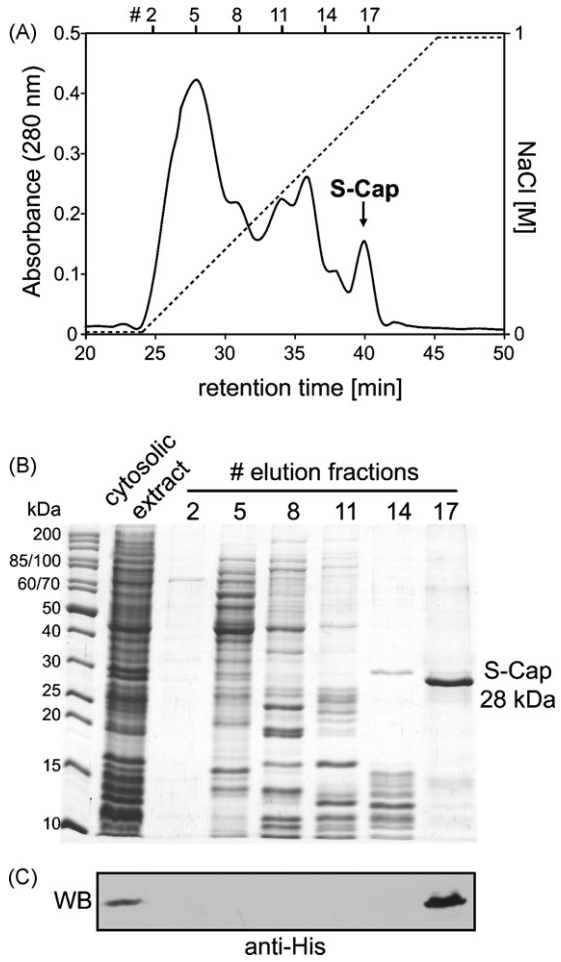


**Fig. 3.** Purification of recombinant S-Cap protein. (A) UnoSphere-S cation-exchange elution profile of cytosolic extract of *E. coli* BL21 λ(DE3) expressing the S-Cap protein. The cytosolic extract was applied onto the column and the bound proteins were eluted with a continuous gradient of NaCl (dotted line). (B) SDS-PAGE analysis of fractions collected from the UnoSphere-S cation-exchange chromatography. M, molecular weight markers (in kDa). Protein profiles of individual elution fractions were indicated by a fraction number. (C) Western blot analysis of fractions collected from the UnoSphere-S cation-exchange chromatography by using anti-His antibody. The blot corresponds to lanes 2–8 from (B).

enriched in the S-Cap protein was recognised by the Cap-positive sera, but not by the pre-immune sera (data not shown). Moreover, a typical dense nuclear staining of PCV 2-infected PK15 cells was seen using the Cap-positive sera compared to the pre-immune sera by *in situ* immunohistochemistry (data not shown). These results documented the immunogenicity of the S-Cap protein isolated under denaturating conditions.

### 3.4. Formation of virus-like particles (VLPs)

The majority of VLPs have been produced using insect or mammalian cell expression systems, but some VLPs have been found to assemble also in *E. coli* cells (Noad and Roy, 2003). In order to detect whether the PCV 2 capsid protein is able to self-assemble into VLPs in *E. coli*, the ultrastructure of the bacterial cells expressing the O-Cap protein, which lacks any of the additional tag (Fig. 1), was examined by using electron microscopy (EM) of ultrathin sections. As shown in Fig. 4, no VLPs were observed in either non-induced cells or *E. coli* cells expressing the O-Cap protein. In contrast, *in vivo* VLPs assembly was detected unambiguously following the expression of a fusion protein containing the capsid and nucleocapsid protein obtained from the Gag polyprotein of Mason-Pfizer monkey virus (Ulbrich et al., 2006) (Fig. 4A and B). In addition, the sucrose density gradient fractionation of cytosolic content of *E. coli* cells expressing O-Cap protein did not reveal any presence of O-Cap protein in the form of VLPs. The absence of VLPs was corroborated also by EM of negatively stained preparations (data not shown). These results demonstrated that the PCV 2 capsid protein was unable to self-assemble into VLPs in the cytosol of *E. coli* cells.

In contrast, self-assembly of the PCV 2 capsid protein into VLPs has been observed repeatedly in baculovirus expression systems (Nawagitgul et al., 2000; Kim et al., 2002; Liu et al., 2008). The VLPs were of similar morphology as the intact PCV 2 virions and appeared to be empty capsids with a less ordered structure (Nawagitgul et al., 2000). However, the question why the PCV 2 capsid protein does form VLPs in insect cells and not in *E. coli* remains to be addressed. Sequence analysis of the PCV 2 capsid protein revealed amino acid residues with consensus patterns for potential post-translational modifications (N-glycosylation, phosphorylation) (Liu et al., 2001b), which are known to occur in eukaryotic but not in prokaryotic expression systems. Another explanation could be a specific need for chaperones, scaffolding proteins, or ssDNA, which might play important roles in a proper PCV 2 assembly (Ludwig and Wagner, 2007). A plausible hypothesis is that the N-terminal portion of the PCV 2 capsid protein is enriched with basic amino acids that could bind preferentially to the bacterial genomic DNA, and therefore, prevent a regular arrangement of the capsid subunits into VLPs. This hypothesis would be supported by the observation that the *E. coli* cells expressing Cap protein produced large amounts of outer membrane vesicles (Fig. 4D) compared to the non-induced cells or the cells expressing the capsid protein of Mason-Pfizer monkey virus. Indeed, the release of outer membrane vesicles has been attributed to a novel envelope stress response (McBroom and Kuehn, 2006), which might result from the binding of the Cap protein to genomic DNA.

### 3.5. Application of S-Cap protein in indirect ELISA

Specificity and sensitivity of both the full-length capsid protein (S-Cap) and its truncated variant (ΔCap-His) to pig PCV 2-positive sera was determined using an indirect ELISA format (Cap-ELISA). Using a chequer-board titration of PCV 2-positive serum (IPMA titre of 5120), optimal antigen concentrations and serum dilutions were selected to be 10 μg/ml and 1:100, respectively. When the positive/negative cut-off value was set to 0.2 (see Section 2), all the PCV 2-positive sera tested were 100% specific (data not shown). Cap-ELISA showed a low background, and significant differences between PCV 2-negative and PCV 2-positive pig sera were detected (Fig. 5). Cap-ELISA did not exhibit any cross-reactivity with pig sera obtained from SPF pigs which were infected experimentally with coronavirus causing a transmissible gastroenteritis (TGE) or a porcine epidemic diarrhoea (PED), respectively. Moreover, no significant differences in specificity and sensitivity of Cap-ELISA were observed between the S-Cap and ΔCap-His proteins when used as coating antigens, indicating that the N-terminus of the capsid protein is not specifically recognised by antibodies in the PCV 2-positive sera (Wu et al., 2008). This showed that the recombinant full-length Cap protein could be used as coating antigen to develop the indirect Cap-ELISA for specific and sensitive detection of PCV 2 antibodies.

PCV2 antibodies are detected currently by indirect immunofluorescence (Ellis et al., 1998), IPMA (Allan et al., 1998), competitive ELISA (Walker et al., 2000) and by indirect ELISA based on either PCV2 viral particles (Nawagitgul et al., 2002) or recombinant PCV 2 capsid protein expressed in baculovirus (Nawagitgul et al., 2002; Blanchard et al., 2003; Liu et al., 2004). However, while both the indirect immunofluorescence and IPMA assays are highly demanding and not suitable for large-scale survey of PCV 2 infection, the

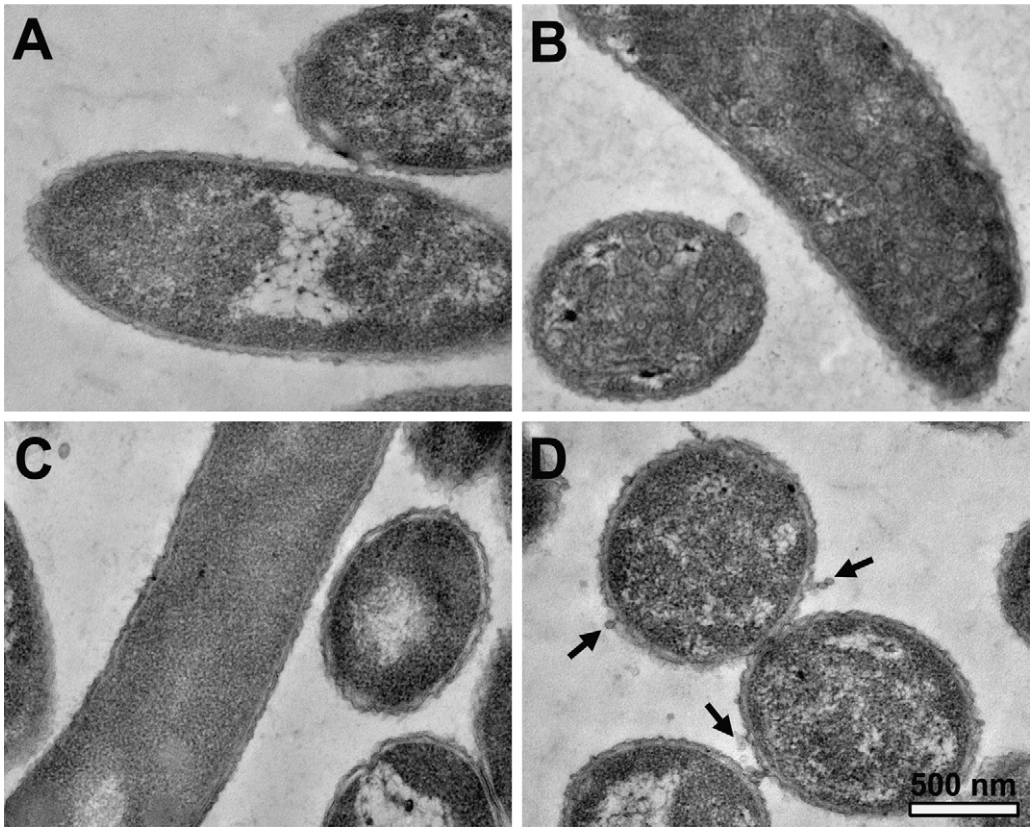


**Fig. 4.** Virus-like particles (VLPs) formation in the cytosol of bacterial cells. Electron micrographs of ultrathin sections of *E. coli* BL21 λ(DE3) expressing a fusion protein of capsid and nucleocapsid protein (CANC) obtained from the Gag polyprotein of Mason-Pfizer monkey virus (Ulbrich et al., 2006) (A and B), and *E. coli* BL21 λ(DE3) expressing the Cap protein (C and D). (A) Non-induced cells, (B) IPTG-induced cells expressing CANC filled with spherical VLPs, (C) non-induced cells and (D) IPTG-induced cells expressing the Cap protein. No VLPs are visible in the cytosol. Note the presence of outer membrane vesicles at the exterior of the cells (arrowheads). Bar, 500 nm.

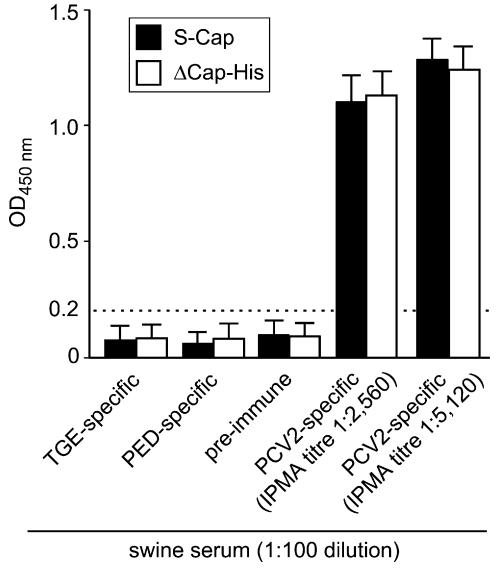


**Fig. 5.** Specificity and sensitivity of the indirect Cap-ELISA. Different pig sera were diluted to 1:100 with PBS and their reactivity with the recombinant S-Cap and ΔCap-His proteins (10 μg/ml) was analyzed by a colorimetric reaction at the optical density at 450 nm ($OD_{450nm}$). $OD_{450nm}$ of 0.2 represents a cut-off value for seropositivity of the samples. TGE-specific and PED-specific pig sera were obtained from SPF pigs which were experimentally infected with the coronavirus causing transmissible gastroenteritis (TGE) and porcine epidemic diarrhoea (PED), respectively. Data represent the means ± S.D. for the three independent experiments.

current ELISA formats are less specific and display antigenic cross-reactivity to non-pathogenic PCV 1 (Magar et al., 2000). Recently, an indirect ELISA based on the recombinant NLS-truncated PCV 2 capsid protein expressed in *E. coli* has been established by Shang et al. (2008). Although a direct comparison in the sensitivity and specificity of the full-length (this study) and the NLS-truncated (Shang et al., 2008) proteins as antigens would be interesting, it is very likely that both ELISA formats are comparable in the detection of PCV 2 antibodies. Considering these data, the Cap-ELISA based on the recombinant full-length Cap protein as coating antigen would provide a simple and reliable tool for standard serodiagnosis of PCV 2 infection.

To further analyze the capacity of Cap-ELISA to detect the PCV 2-specific antibodies, a limited serological and genomic load survey of PCV 2 infection was monitored in piglets during the first 20 weeks of age. The piglet's antibody response along with total amounts of PCV 2 DNA per 1 ml of serum was determined by Cap-ELISA and quantitative PCR, respectively. As shown in Fig. 6A, the levels of PCV 2-specific antibodies showed an initial drop within the first 12 weeks of age, from then on the levels increased gradually until 20th week. In contrast, the DNA copy numbers of PCV 2 increased gradually during the 1st weeks of age to reach the maximum level in the 12th week, after which they decreased slightly until the 20th week (Fig. 6B). These results are in good agreement with the time course dynamics of PCV 2 infection in newborn piglets (McKeown et al., 2005). A dramatic decrease of the PCV 2-specific antibodies in the 12th week of age corresponds to the period in which the passive intake of maternal antibodies from breast milk during the weaning period (weeks 1–8) is discontinued. This results in the onset of PCV 2 infection after the 8th week of age prior to the active production of the intrinsic PCV 2-specific antibodies (weeks 12–20). Taken

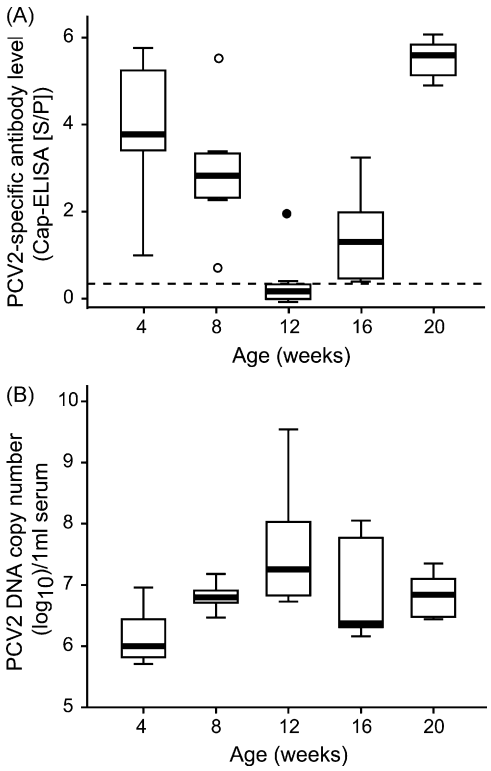


**Fig. 6.** Time course analysis of levels of PCV 2-specific antibodies (A) and DNA copy numbers of PCV 2 per 1 ml of serum (B) in piglets in a herd with PCV2 infection during their first 20 weeks of age. (A) Time course analysis of the PCV 2-specific antibody levels as detected by the indirect Cap-ELISA. The final values were expressed as a signal-to-positive (S/P) ratio. S/P value was determined according to the following formula: ($OD_{450nm}$ of sample − $OD_{450nm}$ of negative control)/($OD_{450nm}$ of positive control − $OD_{450nm}$ of negative control). S/P value of 0.3 represents the cut-off signal for seropositivity of the samples. (B) Time course analysis of the PCV2 DNA copy numbers per 1 ml of serum as detected by the quantitative PCR. Total genomic DNA was extracted from 200 μl of each serum sample and quantified by the PCR amplification of 100 bp DNA segment within the nucleotide sequence of the PCV 2 *cap* gene using a TaqMan format. Results of statistical analysis using one-way analysis of variance (ANOVA) between serum samples obtained at given time intervals from 22 piglets are represented by the box plots. The median value for each dataset is indicated by the black center line. The vertical height of each box indicates the 25–75% data range. The upper and lower bars denote the largest and smallest data values. The marker (○) denotes the extreme value of the individual serum sample that is >1.5 times the inter-quartile range from the upper and lower quartile. The marker (●) denotes the extreme value of the individual serum sample that is >3 times the inter-quartile range from the upper quartile. Data represent the means ± S.D. for the three independent experiments.

together, these data confirmed that Cap-ELISA was developed with a high specificity and sensitivity for detection of PCV 2 antibodies in pig sera and could be used for large-scale surveys of PCV 2 infection at low cost.

## 4. Conclusions

In summary, a bacterial expression system has been developed for the production of the full-length recombinant capsid protein of porcine circovirus type 2. Here, the codon optimization strategy was used to obtain high yields of the recombinant capsid protein in an inexpensive cultivation system. Purification protocol based on the single step cation-exchange chromatography provided a cost effective procedure to obtain substantial quantities of the Cap protein in a high purity. Although the recombinant capsid protein expressed in *E. coli* did not self-assemble into VLPs, the antigenic properties of the Cap protein resembled that of intact PCV 2 virions. In addition, the recombinant full-length Cap protein was used as antigen to develop the indirect Cap-ELISA for specific and sensitive detection of PCV 2 infection.

## Acknowledgement

The excellent technical help of Sona Charvatova, Hana Kubinova, Zuzana Vecerkova and Petra Klodnerova (Proteix, s.r.o.) is acknowledged. We also wish to thank Pavel Ulbrich (Institute of Chemical Technology, Prague, Czech Republic) for providing Mason-Pfizer monkey virus CANC expression vectors. This work was supported by grants GACR 310/07/P115 (L.B.), NPVII 2B06161 (P.S.) of the Ministry of Education, Youth and Sports of the Czech Republic, NPV 1B53016 (I.P.) and 0002716201 (I.P.) of the Ministry of Agriculture of the Czech Republic, and Institutional Research Concept AV0Z50200510 and AV0Z50520701.

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