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Secretory expression of Porcine Circovirus Type 2 capsid protein in *Pichia pastoris*

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Porcine circovirus type 2 (PCV2) is associated with postweaning multisystemic wasting syndrome (PMWS). The PCV2 capsid (Cap) protein is a leading antigen candidate for vaccine and serological diagnostic testing, due to its immunogenic properties. In this study, the codon-optimized PCV2 Cap gene was cloned into a pPICZαA vector for secretory expression in the methylotrophic yeast *Pichia pastoris* after methanol induction. The screening of recombinant yeasts was followed by detection of the recombinant Cap (rCap) protein by Western blot, using sera from pigs naturally infected with PCV2. The rCap secreted protein was used without prior purification as a coating antigen in the ELISA test, with high discrimination between PCV2-positive and negative sera. These results reveal a high confidence in the specific immunoreactivity of the secreted antigen and show the antigenicity of the recombinant protein. The feasibility of the *P. pastoris* expression system for the production of PCV2 Cap as secreted protein and its apparent bioactivity, suggests there are good prospects for the use of this antigen in the investigation of PCV2 infections and testing for vaccine purposes.

1. Introduction

Porcine circovirus type 2 (PCV2) is one of the most important pathogens for swine in many countries (Gillespie et al., 2009). This virus is linked to a set of diseases known as "Porcine Circovirus Associated Diseases" (PCVAD), of which the Postweaning multisystemic wasting syndrome (PMWS) (Opriessnig et al., 2007; Rose et al., 2012) is the most important (Darwich and Mateu, 2012) and has serious economic effects on the swine industry (Marcekova et al., 2009). PCV2 is a small non-enveloped DNA virus with a circular single-stranded genome, and is member of the Circoviridae family (Allan and Ellis, 2000). The PCV2 genome consists of three open reading frames (ORF): ORF1 encodes the Rep proteins involved in viral replication, ORF2 encodes the major capsid protein (Cap) (Nawagitgul et al., 2000) and ORF3 encodes apoptosis-inducing protein (Liu et al., 2006). The PCV2 capsid protein is the primary immunogenic protein (Mahé et al., 2000, Blanchard et al., 2003b). Due to its highly conserved epitopes and induction of a strong immune reaction against sera from PCV2-positive animals (Blanchard et al., 2003a; Wu et al., 2008), this protein has been employed in the development of vaccines and serodiagnostic assays (Patterson et al., 2008; Huang et al., 2011).

Several criteria must be taken into account when making a PMWS diagnosis (Segalés et al., 2005). For instance, it should be noted if the animal shows clinical signs of disease (such as wasting and paleness) and further histopathological lesions as well as PCV2 viral load in the lymphoid tissues (Segalés, 2012). Hence, it is essential to compile a serological profile of animals experiencing PMWS whether they have clinical signs or not (Pinto et al., 2011). Recently, several enzyme-linked immunosorbent assays (ELISA) have been carried out based on recombinant proteins employed as a coating antigen for detecting PCV infection (Wu et al., 2008; Júnior et al., 2009; Yin et al., 2010). The use of recombinant antigens in serological diagnosis has significant benefits because they are cheap and easy to produce, and their antigenicity can be reliably established (Wu et al., 2008).

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In general, yeasts have been consistently employed for heterologous gene expression (Gellisen and Hollenberg, 1997; Sohn et al., 2010). The *Pichia pastoris* expression system has been widely used for the production of recombinant proteins (Schutter et al., 2009), either secreted or in an intracellular form. This system was chosen since it offers several advantages, these include the use of a strong and tightly regulated promoter derived from the alcohol oxidase I (*AOX1*) gene, the ability of the cells to be cultivated at high density and the possibility of introducing typical eukaryotic post-translational modifications in the recombinant protein (Hu et al., 2011). In addition, since *P. pastoris* secretes only a few native proteins, the purification process of the recombinant products can be assisted by employing the secretory pathway (Cregg et al., 2000).

The aim of this study was to obtain the secretory production of PCV2 capsid protein in *P. pastoris* and evaluate the functional activity of the recombinant protein with regard to its antigenic properties.

2. Materials and methods

2.1. Codon optimization of the Cap gene

The heterologous gene was designed on the basis of a sequence of PCV2 BRA1 isolates deposited in the GenBank (DQ364650.1). The PCV2 Cap sequence was synthesized with codons optimized for expression in *P. pastoris*, and a sequence encoding the polyhistidine tag (6xHis) at the C-terminal of the protein was added to allow the immunodetection of the recombinant protein. The optimized sequence was deposited in GenBank with the accession number KM210286. The online program Graphical Codon Usage Analyzer (available at http://gcua.schoedl.de) was used to analyze the codon usage of the heterologous gene and the host organism. The gene was synthesized by Epoch Life Science (TX, USA).

2.2. Cloning of the Cap gene in an expression vector

The codon-optimized Cap gene was digested with *Xho*I and *Not*I enzymes (New Englands Biolabs, MA, USA) and cloned into the pPICZαA expression vector (Life Technologies, SP, Brazil) for secretory expression in the yeast through the use of the α-factor mating secretion signal from *Saccharomyces cerevisiae*, that is present in the vector. *Escherichia coli* DH5α were transformed and the pPICZαA/rCap clones were grown in low salt LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 2% agar) supplemented with 25 μg/mL zeocin™ (Life Technologies). The presence and orientation of the insert were analyzed by enzymatic digestion and DNA sequencing. The sequencing was performed by the dideoxy method (ABI3100 sequencer, Life Technologies), and the alignment of the sequences was carried out by the ClustalW program.

2.3. Transformation of P. pastoris

The recombinant plasmid (10 μg of DNA) was first linearized with the *Sac*I enzyme and used to transform *P. pastoris* strain X-33, phenotype Mut+ (methanol utilization plus) by electroporation, in accordance with the manufacture's instructions (Life Technologies). Briefly, after electroporation, the cells were transferred to a 15 mL sterile tube and incubated for 2 h at 30 °C without agitation. The efficiency of the transformation was increased by adding 1 mL of YPD to the tube, which was incubated for another 2-3 h at 30 °C under agitation. Aliquots of 10, 25 and 100 μL were taken from this material and plated on YPDS medium (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol and 2% Agar) containing 100 μg/mL zeocin and incubated at 30 °C for 5 days.

2.4. Screening of the recombinants (Colony blot and Dot blot)

The recombinants selected by zeocin were screened for rCap protein detection by Colony blot and Dot blot. In the colony blot assay, the plate with the recombinant colonies is incubated with methanol, for induction for 72 h. The PVDF membrane was laid on colonies of the transformants and incubated at 37 °C for 3 h. Following this, the membrane was washed with TTBS, blocked with 5% milk, and incubated with penta-His antibody (Qiagen, SP, Brazil) (1:1000) for 2 h at 28 °C. After further washing stage, the membrane was incubated with the anti-mouse HRP-conjugated antibody (1:2000) (Sigma–Aldrich, SP, Brazil) for 1 h. The reaction was revealed with tetramethylbenzidine (TMB) substrate solution (Sigma–Aldrich). In the case of the dot blot assays, the clones were cultivated on a small scale (5 mL) with YPD medium (1% yeast extract, 2% peptone, 2% glucose) for 24 h. At the end of this period, the clones were induced for heterologous expression by the addition of methanol to a final concentration of 0.5% over a period of 72 h, at 28 °C. The supernatant was recovered and used in the dot blot assay as described by Coimbra et al. (2011) using the monoclonal anti-6xHis antibody conjugated with alkaline phosphatase (1:1000) (Sigma–Aldrich). The NTB/BCIP substrate was used for the revelation of reactions (Sigma–Aldrich).

2.5. Expression of recombinant Cap protein in P. pastoris

P. pastoris transformants were inoculated into 500 mL of BMGY medium (1% yeast extract, 2% peptone, 1.34% YNB, 0.002% biotin, 1% glycerol, 100 mM sodium phosphate, pH 6.0) and incubated at 28 °C for 48 h under vigorous agitation (240 rpm) to obtain biomass. For the induction of the rCap protein, the obtained biomass was centrifuged and transferred to 100 mL of BMMY medium (the same components as those of BMGY with glycerol replaced by 0.5% methanol) in 1 L flasks. Methanol was added to the culture every 24 h to a final concentration of 1%, to maintain the induction of the recombinant protein. The culture was kept at 28 °C with agitation (240 rpm) for 72 h. After this, the cells were centrifuged and the supernatants were recovered.

2.6. SDS-PAGE and Western blot assay

The proteins were concentrated with PEG (Polyethylene glycol) 6000 (10%) (Vetec Quimica Fina, SP, Brazil), boiled at 95 °C for 7 min in SDS-loading buffer (10% 2-mercaptoethanol, 4% SDS, 0.004% bromophenol blue, 20% glycerol, 0.125 M Tris–HCl, pH 6.8) and then separated on 12.5% SDS-PAGE gel. The gel was stained with silver nitrate. For the western blot, two aliquots of the supernatant were used. One was concentrated in PEG, and the other precipitated with 20% trichloroacetic acid (TCA) (Sigma–Aldrich) before being transferred onto PVDF membranes using a Trans-blot Semi-dry apparatus (Scie-Plas, CA, UK) at 320 mA for 1 h.

The membrane was blocked with 5% non-fat dry milk in TBS at room temperature for 1 h and then incubated with swine sera at a dilution of 1:25 at 4 °C for 1.5 h. After three washes in TBS-Tween, the membrane was incubated with peroxidase-conjugated protein G (Sigma–Aldrich) at 4 °C for 1 h. The reaction was revealed using the ECL chemiluminescent kit (GE Healthcare, SP, Brazil). The protein was quantified by means of the Bradford protein assay kit (Bio-rad, SP, Brazil).

2.7. Indirect ELISA using the rCap as antigen (rELISA)

Indirect ELISA was performed to test preliminarily the immunoreactivity of the rCap. The supernatant was dialyzed against PEG 6000 40% (w/v) in phosphate buffered saline (PBS – 0.05 M, 0.15 M NaCl), pH 7.6 through a membrane with a cut-off