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The optimized capsid gene of porcine circovirus type 2 expressed in yeast forms virus-like particles and elicits antibody responses in mice fed with recombinant yeast extracts

Sergio A. Bucarey[a,*], Jorge Noriega[a], Paulina Reyes[a], Cecilia Tapia[b], Leonardo Sáenz[a], Alejandro Zuñiga[c], Jaime A. Tobar[d]

[a] Centro Biotecnológico Veterinario Biovetec, Departamento de Ciencias Biológicas Animales, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Chile
[b] Programa de Microbiología y Micología, ICBM, Facultad de Medicina, Universidad de Chile, Chile
[c] Facultad de Ciencias Físicas Matemáticas, Universidad de Chile, Chile
[d] Investigación y Desarrollo Biológicos, Laboratorio Centrovet, Santiago, Chile

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ABSTRACT

Porcine circovirus type 2 (PCV2)-associated diseases are considered to be the biggest problem for the worldwide swine industry. The PCV2 capsid protein (Cap) is an important antigen for development of vaccines. At present, most anti-PCV2 vaccines are produced as injectable formulations. Although effective, these vaccines have certain drawbacks, including stress with concomitant immunosuppression, and involve laborious and time-consuming procedures. In this study, *Saccharomyces cerevisiae* was used as a vehicle to deliver PCV2 antigen in a preliminary attempt to develop an oral vaccine, and its immunogenic potential in mice was tested after oral gavage-mediated delivery. The *cap* gene with a yeast-optimized codon usage sequence (*opt-cap*) was chemically synthesized and cloned into *Escherichia coli*/*Saccharomyces cerevisiae* shuttle vector, pYES2, under the control of the *Gal1* promoter. Intracellular expression of the Cap protein was confirmed by Western blot analysis and its antigenic properties were compared with those of baculovirus/insect cell-produced Cap protein derived from the native PCV2 *cap* gene. It was further demonstrated by electron micrography that the yeast-derived PCV2 Cap protein self-assembles into virus-like particles (VLPs) that are morphologically and antigenically similar to insect cell-derived VLPs. Feeding raw yeast extract containing Cap protein to mice elicited both serum- and fecal-specific antibodies against the antigen. These results show that it is feasible to use *S. cerevisiae* as a safe and simple system to produce PCV2 virus-like particles, and that oral yeast-mediated antigen delivery is an alternative strategy to efficiently induce anti-PCV2 antibodies in a mouse model, which is worthy of further investigation in swine.

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1. Introduction

Porcine circoviruses type 1 and type 2 (PCV1 and PCV2) are very small (about 17 nm), spherical, non-enveloped viruses with icosahedral symmetry [1]. PCVs have circular, single-stranded DNA genomes of approximately 1700 nucleotides, encoding 2 major ambisense open reading frames, ORF1 and ORF2 [2]. ORF1 is predicted to encode a replicase protein (Rep) essential for replication of viral DNA [3]. ORF2 encodes the capsid protein (Cap), which plays a key role in the process of virus particle assembly [4]. Together with a number of avian viruses with similar molecular characteristics, the porcine circoviruses are classified in the genus *Circovirus* within the family *Circoviridae* [5]. Although PCV1 persists in the pig population, the presence of PCV1 has not been associated with any recognized clinical signs or lesions. In contrast, PCV2 has been implicated as the major causative agent of postweaning multisystemic wasting syndrome (PMWS) [6,7], a disease which is characterized by severe immunosuppressive effects in the porcine host. The latest evidence suggests that PCV2-induced immune disorders are mainly produced by specific silencing of plasmacytoid dendritic cell responsiveness to pathogen-associated danger signals [8]. Therefore, PCV2 infection generates loss of the connection between the innate and acquired immune systems, rendering a host susceptible to secondary or concomitant microbial infections [8]. Consequently, PCV2 is also associated with many other diseases such as respiratory disease complex, reproductive failure, porcine dermatopathy and nephropathy syndrome (PDNS), congenital tremor, nectrotizing tracheitis and exudative epidermitis [9–12]. These

* Corresponding author at: Biotechnological Veterinary Center, Biovetec Departamento de Ciencias Biológicas Animales, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile. Santa Rosa 11735, La Pintana, Santiago, Chile.
Tel.: +56 2 9785658; fax: +56 2 9785659.
E-mail address: sbucarey@uchile.cl (S.A. Bucarey).

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diseases are known as PCVAD or PCV2-associated diseases, a name that the American Association of Swine Veterinarians (AASV) uses to group together all the diseases attributed to porcine circovirus type 2, including PMWS [13].

PCV2-associated diseases are considered to be the biggest economic problem of the pig industry worldwide [13]. In addition to improved management and husbandry practices (better hygiene, less overcrowding and better ventilation), the availability of several anti-PCV2 vaccines represents an efficient immunological option to blunt the diseases' impact [14]. At present, four vaccines against PCV2 have been introduced in the international market. All of these vaccines are non-replicative (sub-unit or inactivated) and most contain the PCV2 capsid protein as the immunogenic antigen. In all cases, reports from field trials suggest that commercially available PCV2 vaccines significantly contribute to decreased mortality rates and to improved pig growth in PMWS-affected farms [15,16], thereby reducing the economic impact that PCVAD has on pig production worldwide.

The Cap protein is the major immunogenic protein of the virus and the principal carrier of type-specific epitopes [17]. Recently, multiple *in vitro* expression system strategies have been investigated in order to produce Cap protein as an immunogenic PCV2 subunit vaccine, including bacterial-vector vaccines (*Escherichia coli*) and recombinant virus-vector vaccines (baculovirus, pseudorabies virus) [18–20]. Although each system has unique features and advantages, each system also has limitations that prevent further development of the recombinant protein into a useful subunit vaccine. Bacterial expression is simple and provides high yields, but the effectiveness of the immune response is dependent on correct protein folding and post-translational modification, which are variable and are determined by the nature of the antigen.

In contrast, the baculovirus/insect cell expression system is highly effective for heterologous expression of multiple viral structural proteins, supporting their self-assembly into virus-like particles (VLPs) [21–23]. At present, two baculovirus-based PCV2 subunit vaccines are frequently used with successful results; however, these vaccines are time-consuming to administer and relatively expensive.

Saccharomyces cerevisiae (*S. cerevisiae*), a non-invasive, non-pathogenic organism, is generally considered to be a generally regarded as safe (GRAS) species and for this reason, it is an attractive tool for delivering antigens and therapeutic molecules [24,25]. *S. cerevisiae* used as a vaccine vehicle can effectively elicit mucosal and systemic immunization by administration to mucosal sites, including the oral, respiratory and genital tracts [26–31]. In mammals in particular, *S. cerevisiae* has been demonstrated to have superior safety and efficacy, where it has been shown to elicit both innate and adaptative immune responses due to the presence of TLR ligands in its cell wall [32].

Yeast expression systems provide an alternative mechanism for capsid protein expression. As a eukaryote, yeast has many advantages. Proteins produced by yeast are likely to be processed with proper folding and chemical modifications, and there are several reports which confirm that yeast can support VLP auto-assembly of viral proteins [33–36]. This ability enhances recognition of the expressed antigens by the host as native antigens, improving the quality of the induced immune response [26,28,33–36]. Furthermore, yeast cultures are easy to maintain, which makes them faster and less expensive to use than other eukaryotic expression systems, such as insect or mammalian cell cultures.

To date, all anti-PCV2 vaccines on the market have been produced as injectable formulas. Although injectable vaccines are efficient, there are problems associated with the use of injectable products, including laborious and time-consuming procedures, the induction of inflammatory responses at the injection site, and management-derived stress in the animals [37,38]. Oral vaccines represent an improvement on antigen-delivery technology; they overcome the problems associated with injection management and facilitate antigen boostering when the animal's immunity is below the protective window [32,39,40]. Several oral delivery systems have been attempted using attenuated bacterial mutants [41], biopolymer-based microparticles [42], liposomes [43], recombinant plants [44], and yeast [33]. In this report, the expression of a PCV2 synthetic capsid protein gene, its codon optimization for expression in yeast, and its potential application as a PCV2 oral vaccine are presented and discussed. Specifically, the "edible vaccine" concept is probed by studying the safety of minimally purified materials using oral gavage-mediated vaccine delivery. The results show that the yeast-derived PCV2 Cap self-assembles into VLPs that are morphologically and antigenically similar to insect cell-derived PCV2-VLPs, and that the expressed protein is recognized by a PCV2-specific antibody and serum from PCV2-vaccinated and PMWS-affected pigs. It is also demonstrated that yeast-derived recombinant VLPs are immunogenic in mice, and that oral administration of raw yeast extracts induces significant serum as well as fecal antibody responses to PCV2. Thus, we conclude that it is feasible to use *S. cerevisiae* as a simple and safe system to produce virus-like PCV2 particles that orally induce anti-PCV2 specific antibodies in a mouse model. These results support the future study of a recombinant Cap yeast strain as an anti-PCV2 oral vaccine in the pig, the natural host for PCV2.

2. Materials and methods

2.1. Experimental design and animals

Five-week-old Balb/C female mice were obtained from the Faculty of Veterinary Sciences at the University of Chile. The animals were randomly distributed in three experimental groups (5 mice/group) and were maintained with *ad libitum* access to food and water in a temperature and light-controlled environment. Two groups were designated to evaluate the specific anti-PCV2 antibody response in serum (IgG/IgM) and in faeces (IgA), against baculovirus/sf9-produced PCV2 VLPs, by using an in house-developed PCV2-VLP-based indirect ELISA, after oral administration of raw extracts of *S. cerevisiae* expressing the yeast-optimized *cap* gene (*S. cerevisiae*/pYES2::*opt-cap*, group 1) or *S. cerevisiae* with an empty plasmid (*S. cerevisiae*/pYES2, group 2), which were compared against an untreated group (group 3). Because the untreated group present similar responses than mice immunized with *S. cerevisiae*/pYES2, only data compared with *S. cerevisiae*/pYES2 administration are presented in all experiments. Briefly, 150 mg yeast extract powder was dissolved in 1 ml of PBS and orally administered at a volume of 200 μl per mouse ($\sim$30 mg per mouse) through oral gavage five times with a 15-day interval between treatments. The concentration of PCV2 Cap protein in each dose (approximately 100 μg) was determined by Western blot analysis using serial dilutions of bacterially produced Cap-6xhis fusion protein, as described below. Blood and faecal samples were collected before the first administration and at days 15, 30, 45, 60, 75 and 90 after the first administration. Blood was collected, clotted at room temperature, left to stand at 4 °C overnight, and then centrifuged at 2500 $\times$ *g* for 20 min at 4 °C. Sera were collected and stored at −20 °C until use. For IgA analysis, 100 mg of feces was suspended in 1 ml PBS (20%, w/v), vortexed, and then centrifuged for 10 min at 10,000 $\times$ *g* to remove debris. The supernatants were collected and stored at −20 °C until further analysis.

An additional experimental group (three mice) was designated to confirm the immunogenicity elicited by yeast-derived antigens administered subcutaneously (positive control for ELISA test). For this experiment, PCV2 Cap protein extracts prepared as described

above were emulsified in one volume of complete Freund's adjuvant (Sigma). 100 μg of protein extract, equivalent to 0.1 ml of formulated vaccine, was then injected subcutaneously and a boosting immunization with the same amount and type of antigen emulsified with incomplete Freund's adjuvant (Sigma) was administered at 2 weeks after the first immunization. A third and final immunization was performed by the same route 2 weeks after boosting immunization with the same booster formula [45]. Blood was drawn to collect serum 5 days after the final boosting immunization. Finally, IgG/IgM antibody response assays were carried out to confirm the immunogenicity of the yeast-derived antigen (data not shown).

The animals were anesthetized with a mixture of isofluorane/O_2 for blood extraction and vaccination. This protocol was approved by the institutional animal bioethics committee, as stipulated in the guide to the care and use of experimental animals of the Canadian Council on Animal Care (CCAC).

2.2. Cloning and sequence analysis of the native PCV2 cap gene

A fragment encompassing the full-length PCV2 *cap* gene (ORF2) was amplified using DNA from swine lymph nodes obtained from a Chilean PMWS case–control study (unpublished results) as a template, with the following PCV2 type-specific primers: PCV2 ORF2, 5′-CCCAAGCTTCGATGACGTACCCAAGGAGGCG-3′ and PCV2 ORF2-3, 5′-CGGGGTACCTTATGGTTTAAGTGGGGGGTC-3′. The products were then cloned into the pGEM-T vector (Promega) and sequenced. The nucleotide sequences obtained were analyzed using Vector NTI software (Invitrogen) and published in the GenBank database under the following accession numbers: EU750909, EU519224, EU519223, and EU186062. All the sequences correspond to PCV2 genotype "a" (PCV2a). One of these clones (EU519223) was subsequently subcloned in the expression vectors pYES2 (Invitrogen), pFastBac (Invitrogen) and pQE80L (Qiagen) in order to study the immunogenic properties of PCV2 Cap protein produced in yeast, insect cells, and bacteria, respectively.

2.3. Codon optimization and opt-cap gene synthesis

The entire open reading frame of the PCV2 *cap* gene (Genbank EU519223) was codon-optimized for *S. cerevisiae* codon usage. For this, the predicted amino acid sequence was taken from the Entrez database and back-translated to an *S. cerevisiae*-optimized nucleotide sequence using Vector NTI® software (Invitrogen). The algorithm utilizes a matrix for the most frequently occurring codons in *S. cerevisiae* and also removes cryptic splice sites, RNA destabilizing sequence elements, and adjusts the GC content. The *opt-cap* gene was synthesized and provided by GenScript Corporation (Piscataway, NJ). Two stop codons were added at the C terminus to form a termination signal. For further cloning steps, flanking BamHI and NotI restriction sites were introduced. In order to place the synthetic DNA in frame with the expression vector ribosomal binding site, it was necessary to add two nucleotides immediately after the N-terminal BamHI restriction site. Finally, the construct was assembled from synthetic oligonucleotides and cloned into the pYES2 vector (Invitrogen) using the BamHI and NotI restriction sites. The pYES2::*opt-cap* plasmid DNA was purified (Pure Yield Plasmid Midiprep, Qiagen) from transformed bacteria (*E. coli* Top 10), and its sequence identity was confirmed.

2.4. S. cerevisiae transformation, gene expression conditions and recombinant yeast extract preparation

The pYES2, pYES2::*cap* and pYES2::*opt-cap* plasmids were transformed into the expression host *S. cerevisiae* INVSc1 (genotype: MATa his3Δ1 leu2 trp1-289 ura3-52/MATα his3Δ1 leu2 trp1-289 ura3-, phenotype: His-, Leu-, Trp-, Ura-) using the LiAc/SS carrier DNA/PEG method [46]. The transformed colonies were cultured using selective autotrophic YNB URA medium (yeast nitrogen base 6.7 g, casamino acids 5 g, glucose 20 g, tryptophan 0.03 g, and bacto-agar 20 g in 1000 ml of distilled water) for 48 h at 30 °C. The recombinant colonies were picked and transferred into 10 ml of liquid YNB URA medium and cultured overnight at 30 °C until they reached a density of 0.6–0.7 at OD_{600}. The cells were harvested and, after washing twice with PBS, inoculated into 50 ml of induction medium (YNB URA medium containing 2% galactose instead of glucose). The cells were induced by 24 h of incubation at 30 °C with shaking, and were harvested by centrifugation at 1500 × *g* for 5 min at 4 °C and resuspended in 5 ml of PBS (pYES2 Manual, catalog no. V825-20, Invitrogen). Then, the cells were sonicated on ice for five 60 s cycles with 20 s intervals using a Branson Digital Sonifier® operated at 40% amplitude. The raw extracts were lyophilized and analyzed by SDS-PAGE and Western blotting using a mouse anti-Cap PCV2-specific monoclonal antibody, isotype IgG2a (Jeno Biotech Inc., Republic of Korea). The raw extracts were ground to make the yeast powder used for the oral immunization experiments.

2.5. Measurement of Cap-specific antibody response in immunized mice

Anti-Cap antibodies (IgG, IgM and sIgA) in sera were measured using a PCV2-VLP-based indirect ELISA previously validated by SERELISA® PCV2 Ab Mono Blocking (Synbiotics Corporation). 40 μg of sf9-produced Cap protein (below) dissolved in 100 μl of coating buffer (14.2 mM Na_2CO_3, 34.9 mM $NaHCO_3$, 3.1 mM NaN_3, pH 9.6) was added to a 96 well microplate (MaxiSorp, Nunc) and incubated overnight at 4 °C. Plates were washed three times with PBST, blocked with PBST containing 1% bovine serum albumin (BSA), and incubated overnight at 4 °C for 1 h. For the first antibody, 1:100 diluted sera collected from immunized mice and 1:10 diluted fecal extract were used for IgG/IgM and sIgA analysis, respectively. 100 μl of diluted primary antibody was then added to the plate and incubated overnight at 4 °C. After washing five times with PBST, 100 μl of goat anti-mouse IgG (H + L), anti-mouse IgM (μ-chain specific), or anti-mouse IgA (α-chain specific)-HRP conjugate (KPL) was added to the plate and incubated for 1 h at 37 °C. After washing five times with PBST, the signal was detected by adding 100 μl of the colorimetric substrate tetramethyl benzidine (TMB) in a one step solution as directed by the manufacturer (Pierce, Rockford, IL). After a 20 min incubation at room temperature, the OD was measured at 450 nm using a microplate reader (BioRad). Serum from mice immunized by subcutaneous injection of yeast-produced PCV2 VLPs mixed with Freund's adjuvant was used as a positive control (above). Color development of positive control wells was standardized to allow comparison of results from experiment to experiment. The serum dilutions used for ELISA data analysis yielded ODs in a linear range. Serum from naïve mice was used as a negative control. All samples were run in triplicate. Results are expressed as the means ± standard deviations (SD) of the optical densities.

2.6. Western blot analysis of yeast induction processes

10 mg of yeast extracts from the induction processes were resolved by 12% SDS-PAGE electrophoresis [47] under reducing conditions (1% glycerol, 0.4% SDS, 0.1% mercaptoethanol, 12.5 mM Tris–HCl, pH 6.6) and were electrotransferred onto a nitrocellulose membrane (BioRad) using a TransBlotTM Semi-Dry Transfer Cell (BioRad, USA). The nitrocellulose membrane was blocked overnight with 5% skim milk at 4 °C, incubated overnight at 4 °C with a 1:100 dilution of a mouse anti-Cap PCV2-specific monoclonal antibody (isotype IgG2a, Jeno Biotech Inc., Republic of Korea) in PBS-0.1% Tween-20 (PBST), or with hyperimmune swine serum obtained

from pigs vaccinated against PCV2 with the inactivated VLP-based vaccine (Ingelvac® CircoFLEX™, Boehringer Ingelheim Vetmedica GmbH). After washing with PBST, the membrane was incubated with a goat anti-mouse IgG (H + L)-horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories Inc.) or goat anti-swine IgG (H + L)-horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories Inc.) for 1 h. After further washing, the signal was detected using the colorimetric substrate 4-chloro-1-naphthol/H_2O_2 as directed by the manufacturer (Pierce, Rockford, IL). High-Range Molecular Weight Markers (New England Biolabs) were used on Western blot membranes. 10 mg of lyophilized raw extract of *S. cerevisiae*/pYES2 was used as a negative control. The concentration of yeast-produced Cap protein was estimated by comparing Western blot signal intensities to those in a serial dilution of highly purified bacterial-produced 6xhis-Cap fusion protein preparation of known concentration by densitometry analysis, as described by [28].

In addition, 10 μg of highly purified bacterial-produced 6xhis-Cap fusion protein and 10 μg of baculovirus-produced Cap protein were compared with yeast-produced Cap protein using similar Western blot analysis to that described above.

2.7. Cap protein expressed in insect cells

In order to compare the antigenic and structural properties of yeast-produced Cap protein with an established source of PCV2 virus-like particles (VLPs), Cap was expressed in a baculovirus/Sf9 insect cell system, and the PCV2-VLPs produced were used as the antigen in a PCV2 indirect ELISA. Wild-type Autographa california nuclear polyhedrosis virus (wt.AcNPV) and recombinant AcNPV expressing Cap protein (Ac.Cap) stocks were prepared according to the manufacturer's protocol (Bac-to-Bac® Baculovirus Expression System, Invitogen). *Spodoptera frugiperda* (Sf9) cells were used to propagate baculovirus and were cultured in serum-free Grace's insect medium (Gibco) at 27 °C. Cap sequence was digested with KpnI and PstI, and cloned into the corresponding sites of the baculovirus transfer vector pFastBac™ (Invitrogen). Recombinant baculovirus carrying the *cap* gene (Ac.Cap) was constructed as described previously [4]. Briefly, transfer vector was transformed into *E. coli* DH10Bac (Invitrogen) containing a baculovirus shuttle vector (bacmid) and helper vector. Within *E. coli* DH10Bac, the *cap* gene was transposed into the bacmid. The *E. coli* colonies containing recombinant bacmid (rBac.Cap) were collected three times by blue/white selection. After isolation and purification, rBac.Cap DNA was transfected into Sf9 cells to yield recombinant baculovirus Ac.Cap. Sf9 cells were infected with wt.AcNPV and Ac.Cap at a multiplicity of infection of 10 plaque-forming units per cell. The cells were harvested at 72 h post-infection, and supernatants were purified by sucrose gradient ultracentrifugation as described below and analyzed by SDS-PAGE. Expression of Cap protein in the purified supernatant was confirmed by Western blotting using a mouse Cap PCV2-specific monoclonal antibody isotype IgG2a (Jeno Biotech Inc., Republic of Korea).

2.8. Bacterial Cap protein preparation

The complete PCV2 capsid protein gene was subcloned in a pQE80L expression vector (Qiagen Inc., USA), using SphI and KpnI restriction sites to generate an in frame genetic fusion with the polyhistidine tag of the plasmid. The bacterial Cap protein was used to produce a fusion protein, 6xhis-Cap. Briefly, the recombinant *E. coli* strain BL21 (Amersham) containing the pQE80L::*cap* plasmid was grown in Luria Broth medium (10 g/l yeast extract, 16 g/l tryptone, 5 g/l NaCl, 100 μg/ml ampicillin, pH 7.0) and induced by a final concentration of 0.1 mM isopropylthio-b-D-thiogalactoside (IPTG) for 5 h at 37 °C. The cells were pelleted and resuspended in lysis buffer (8 M Urea, 10 mM Tris, 100 mM NaH_2PO_4, 1% Triton X-100, pH 8.0) and then lysed by sonication on ice for two 60 s cycles using a Branson Digital Sonifier® operated at 10% of amplitude. After centrifugation at 10,000 × *g* for 10 min at 4 °C, the supernatant was loaded on a Ni-NTA affinity column (Ni-NTA Purification System, Invitrogen) according to the manufacturer's protocol. After washing twice with PBS, Cap protein was eluted with an elution buffer (50 mM Tris–HCl, 10 mM Imidazol reduced, pH 8.0) and collected. The collected samples were analyzed by SDS-PAGE and Western blot assays as described below. The concentration of Cap protein was determined using a Coomassie (Bradford) Protein Assay Kit (Pierce, Rockford, IL).

2.9. Purification of virus-like particles

500 μl of insect cell supernatant or clarified yeast extract expressing Cap protein was layered on a sucrose discontinuous gradient (20–50%) and centrifuged at 20,000 × *g* for 18 h using a Beckman SW-28 rotor. The gradients were fractioned by bottom puncture of the centrifuge tube and approximately 10 fractions were collected. The fraction densities were determined using a refractometer 32-G110e (Carl Zeiss Jena, Germany). Fractions with densities between 1.2 and 1.27 g/cm^3 (three) were pooled and the presence of Cap protein in the fractions was determined by SDS-PAGE. The VLPs preparations were dialyzed against PBS and stored at −20 °C until further use.

2.10. Transmission electron microscopy (TEM)

20 μl of insect cell- and yeast-produced VLPs preparations were diluted 1/10, adsorbed onto a carbon-coated copper grid, and incubated for 5 min. Then, the grids were dried using filter paper, negatively stained with 3% of phosphotungstic acid (PTA) for 5 min, and viewed using a transmission electron microscope, a TEM Zeiss EM 109, operating at 80 kV.

2.11. High-resolution transmission electron micrographs (HR-TEM)

For high-resolution transmission electron microscopy studies, purified preparations of yeast-produced VLPs were diluted 10 times and adsorbed onto carbon-coated copper grids for 5 min. Then, the grids were dried using filter paper, negatively stained with 3% PTA for 5 min, and viewed using a FEI Tecnai F20 FEG TEM operating at 200 kV. The HR-TEM studies were performed at the Laboratorio de Microscopía Electrónica de Transmisión, Facultad de Ciencias Físicas y Matemáticas, Universidad de Chile.

2.12. ELISA immunoreactivity analysis of yeast-produced Cap protein with anti-PCV2 swine sera

An ELISA using purified yeast-produced PCV2 VLPs as an antigen was utilized to test whether yeast expressing Cap protein possess the same antigenic properties as the commercial VLP-based anti-PCV2 subunit vaccine. Yeast-produced Cap protein (150 ng/well) was coated on 96-well microtiter plates and incubated at 4 °C overnight. After blocking with 5% skim milk, various serial dilutions of serum samples from pigs vaccinated with commercial anti-PCV2 vaccine (above) were added to the wells and the plates were then incubated at 37 °C for 1 h. After washing with 0.05% Tween 20-PBS, the bound serum antibodies associated with IgG were detected by adding the corresponding secondary antibody goat anti-swine IgG (H + L)-horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories Inc.) for 1 h at 37 °C. HRP activity was then measured with a TMB in a one step solution as directed by the manufacturer (Pierce,

Rockford, IL) and the OD_{450} values were read with a microplate reader (BioRad).

2.13. Source of swine anti-PCV2 sera

This study was conducted using serum samples from hybrid pigs obtained from commercial cross breeds (Landrace (f) × Pietrain (m)) on a Chilean pig farm. The pigs were routinely vaccinated against PCV2 with the inactivated VLP-based vaccine (Ingelvac® CircoFLEX™, Boehringer Ingelheim Vetmedica GmbH). The vaccine was administered intramuscularly as a single 1 ml dose in the right neck region when piglets were 21 ± 3.18 days (mean ± S.D.) old, according to the Ingelvac® CircoFLEX™ Injection Guide (www.bi-vetmedica.com). Blood was collected at days 10, 30, 50, 70, 90 and 110 after vaccination, and serum was stored at −20 °C. The Ingelvac vaccine contained the PCV2 VLP-forming Cap as the active component and an aqueous polymer (carbomer) as an adjuvant. For vaccine production, the *cap* sequence from a North American PCV2 isolated from the tonsils and livers of two pigs with signs of PMWS was used [16].

2.14. Statistical analysis

All results are expressed as means ± standard deviations of five mice per treatment group. Differences between groups and over time were analyzed using one-way (repeated measurement) ANOVA with Tukey's post-test. Differences were considered to be significant if probability values of P were <0.01(*) or <0.05 (**). These tests were performed using STATISTICA® software.

3. Results

3.1. Expression of yeast-optimized cap gene (opt-cap) in S. cerevisiae

The initial study involved transforming *S. cerevisiae* strain INVSc1 with the native PCV2 *cap* gene cloned into the pYES2 vector. This system resulted in low transformation, abnormal colony formation, reduced yeast viability, and almost undetectable amounts of Cap protein expression, probably due to DNA toxicity. To optimize the yield of Cap protein expressed by yeast, the entire PCV2 *cap* open reading frame was codon-optimized to *S. cerevisiae* codon usage, resulting in *opt-cap* gene (Fig. 1). The *opt-cap* sequence was artificially synthesized and cloned into the pYES2 vector. In contrast to pYES2::*cap*, yeast transformed with pYES2::*opt-cap* showed a high transformation efficiency and normal growth rates, suggesting that circovirus codon usage or the DNA structure in the original *cap* gene acts as a limiting factor for yeast survival and/or protein expression (unpublished results).

Intracellular expression of the *opt-cap* gene was detected by Western blot analysis of yeast extract using an anti-Cap PCV2 specific antibody. This revealed a specific 30 kDa band, corre-

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Optimized    1  ATGACATACCCACGTAGGCGTTACCGTCGCCGTCGCCACAGACCTCGTTCCCACCTCGGT
Original     1  ATGACGTATCCAAGGAGGCGTTACCGGAGAAGAAGACACCGCCCCCGCAGCCATCTTGGC

Optimized   61  CAGATCCTGCGCCGCCGTCCATGGCTGGTTCACCCTAGGCACCGTTACCGCTGGAGGCGC
Original    61  CAGATCCTCCGCCGCCGCCCCTGGCTCGTCCACCCCCGCCACCGTTACCGCTGGAGAAGG

Optimized  121  AAGAATGGAATCTTCAACACCCGCCTGTCAAGAACCTTTGGTTATACGATCAAACGTACG
Original   121  AAAAATGGCATCTTCAACACCCGCCTCTCCCGCACCTTCGGATATACTATCAAGCGAACC

Optimized  181  ACCGTCAAGACCCCATCTTGGGCCGTAGACATGATGAGGTTCAACATCAACGACTTTCTT
Original   181  ACAGTCAAAACGCCCTCCTGGGCGGTGGACATGATGAGATTCAATATTAATGACTTTCTT

Optimized  241  CCCCCAGGCGGAGGTAGCAACCCCAGGTCAGTCCCGTTCGAGTACTACAGAATCCGCAAG
Original   241  CCCCCAGGAGGGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATACTACAGAATAAGAAAG

Optimized  301  GTGAAAGTTGAGTTTTGGCCCTGCTCCCCCATCACTCAAGGTGACAGAGGAGTCGGATCA
Original   301  GTTAAGGTTGAATTCTGGCCCTGCTCCCCGATCACCCAGGGTGACAGGGGAGTGGGCTCC

Optimized  361  TCCGCAGTCATCTTGGACGACAACTTCGTGACCAAGGCAACGGCCCTGACCTACGACCCA
Original   361  AGTGCTGTTATTCTAGATGATAACTTTGTAACAAAGGCCACAGCCCTCACCTATGACCCC

Optimized  421  TACGTCAACTACAGCTCCCGCCATACCATCACACAGCCTTTCTCATACCACTCCCGTTAC
Original   421  TATGTAAACTACTCCTCCCGCCATACCATAACCCAGCCCTTCTCCTACCACTCCCGCTAC

Optimized  481  TTTACGCCTAAACCCGTCCTCGACTCAACCATCGACTACTTCCAGCCAAACAACAAGAGG
Original   481  TTTACCCCCAAACCTGTCCTAGATTCCACTATTGATTACTTCCAACCAAACAACAAAAGA

Optimized  541  AACCAACTGTGGTTGAGGCTCCAGACGGCCGGCAACGTCGATCACGTCGGCCTGGGTACT
Original   541  AATCAGCTGTGGCTGAGACTACAAACTGCTGGAAATGTAGACCACGTAGGCCTCGGCACT

Optimized  601  GCCTTCGAGAACAGTATCTACGACCAGGAATACAACATCCGTGTTACAATGTACGTGCAG
Original   601  GCGTTCGAAAACAGTATATACGACCAGGAATACAATATCCGTGTAACCATGTATGTACAA

Optimized  661  TTCCGTGAATTCAACCTCAAAGACCCCCCTTTGAACCCA
Original   661  TTCAGAGAATTTAATCTTAAAGACCCCCCACTTAACCCT
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Fig. 1. PCV2-*cap* gene and yeast-optimized *cap* gene alignment. The changed codons are indicated as red letters (401-mer). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

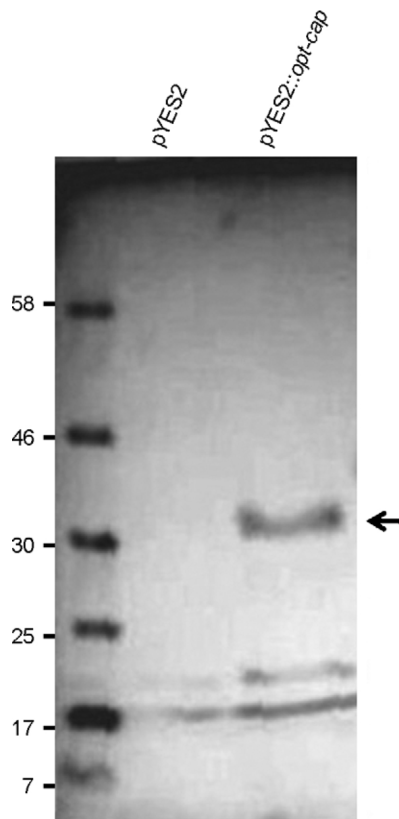

Fig. 2. Western blot analysis of the PCV2 Cap protein expressed in yeast cells. Total lysate from *S. cerevisiae* cells transformed with pYES2-empty or pYES 2 expressing the *opt-cap* gene were loaded and separated on an SDS-PAGE gel, transferred to a nitrocellulose membrane, probed with mouse anti-Cap PCV2 specific antibody, and detected with HRP-conjugated goat anti-mouse IgG. The arrow shows the expected size of Cap ∼30 kDa.

sponding to that of the PCV2 Cap protein. The band was present only in *S. cerevisiae*/pYES2::*opt-cap* cell extracts and not in the extracts of *S. cerevisiae* expressing the pYES2 control vector (Fig. 2).

3.2. Purification of yeast-produced Cap protein

To purify the Cap protein, recombinant *S. cerevisiae*/pYES2 *opt-cap* cells were induced by incubating them for 24 h with galactose and then lysing them by ultrasonic disruption. The clarified cell extracts were ultracentrifuged through a sucrose gradient and multiple protein fractions were obtained. Protein fractions were separated on an SDS-PAGE gel and stained with Coomassie blue. After staining, one main band of approximately 30 kDa was visible in the second and third fraction (Fig. 3A). According to the molecular data for PCV2 Cap protein [2], the sizes of these bands seemed to corresponded to that of Cap protein. We tested this by comparing the electrophoretic mobility and Western blotting analysis of yeast-produced Cap protein with two other sources of purified Cap protein, a highly purified bacterially produced 6xhis-Cap fusion protein and baculovirus/sf9-produced Cap protein. The results showed that the yeast-produced Cap protein exhibited the same electrophoretic mobility as *E. coli*-produced 6xhis-Cap fusion protein (Fig. 3B). However, a shift in mobility was detected by SDS-PAGE and blotting analysis of PCV2 Cap protein expressed in sf9 cells (Fig. 3C). The shifted protein also corresponded to Cap protein, as determined by antibody-specific Western blotting (Fig. 3C).

3.3. Antigenic properties of yeast-produced Cap protein

The antigenicity of yeast-derived Cap protein was studied using sucrose gradient-purified Cap protein preparations. Hyperimmune serum from pigs immunized with VLP-based commercial anti-PCV2 vaccine (Ingelvac® CircoFLEX^{TM}, Boehringer Ingelheim Vetmedica GmbH) reacted with yeast-derived Cap protein in both Western blotting and ELISA analysis (Fig. 4). In addition, yeast-produced Cap protein detected specific serum responses in pigs with naturally occurring PMWS (data not shown), indicating similar antigenic characteristics to those of native virions. These experiments were designed under the rationale that, if the antigenicity of PCV2 protein remained after codon optimization and heterologous yeast expression, it would react with antibodies from pigs immunized with an established source of PCV2 antigen. Fig. 4 shows that swine serum reacted with the yeast-derived Cap protein at various time-points after immunization as shown by Western blot analysis and ELISA (Fig. 4A and B), suggesting

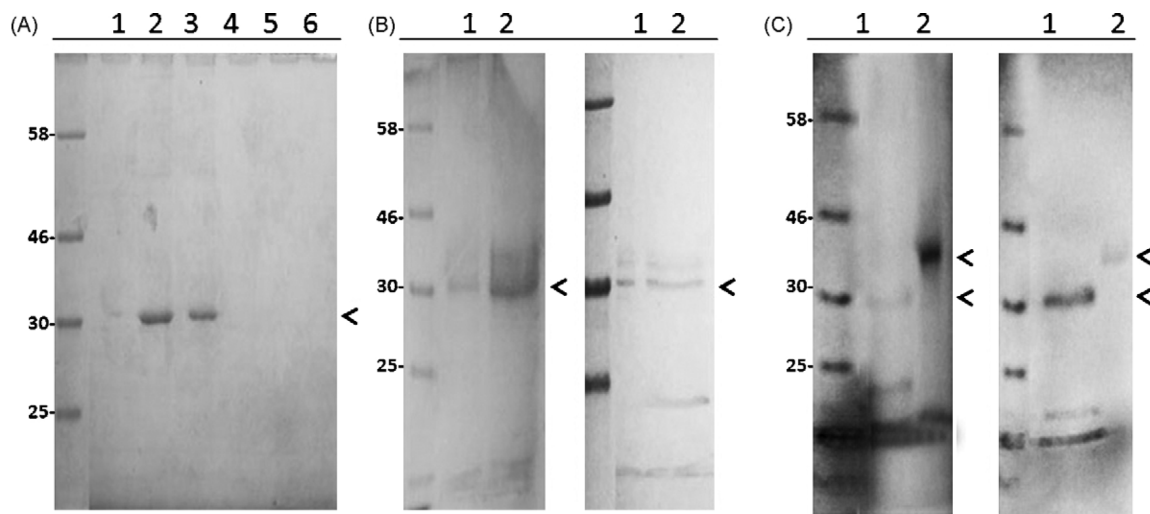

Fig. 3. Purification and mobility analysis of PCV2 Cap protein produced in yeast, bacteria and insect cells. Panel A, purified fractions of *S. cerevisiae*/pYES2::*opt-cap* extract (lanes 1–3), and control *S. cerevisiae*/pYES2 (lanes 4–6) were separated on an SDS-PAGE gel and stained with Coomassie blue. Panels B and C, comparative SDS-PAGE (left) and Western blot (right) analysis of the PCV2 Cap protein expressed in different recombinant systems. B, Cap protein expressed in *S. cerevisiae* (lane 1) and bacteria (lane 2). C, Cap protein expressed in *S. cerevisiae* (lane 1) and in sf9 cells (lane 2). The arrows show the observed mobility of Cap protein in each recombinant system.

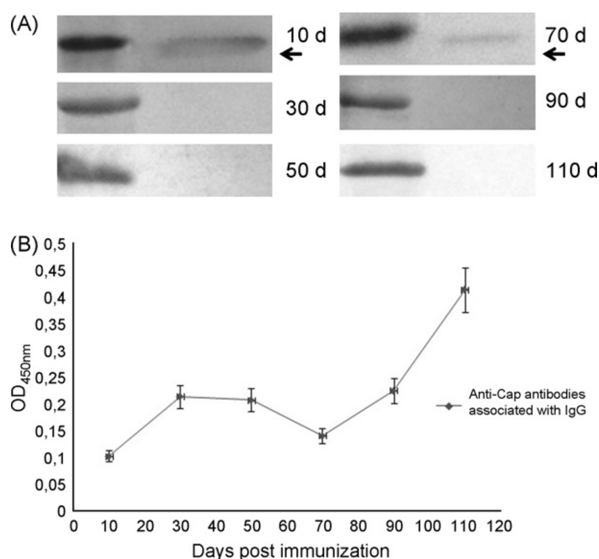

Fig. 4. Immunogenic reactivity of yeast-produced Cap protein with swine serum obtained at multiple time-points after immunization. Panel A, purified yeast-produced Cap protein was loaded and separated on an SDS-PAGE gel, transferred to a nitrocellulose membrane, probed with vaccinated swine serum as the primary antibody, and detected with HRP-conjugated anti-swine IgG. Panel B, indirect ELISA was performed using purified yeast-produced Cap protein as the antigen, which was subsequent incubated with vaccinated swine serum as the primary antibody and detected with HRP-conjugated goat anti-swine IgG.

that the PCV2 vaccine elicits antibody responses against both the conformation-dependent and linear epitopes present in Cap protein. This conclusion is supported by the finding that denaturing SDS-PAGE-Western blot analysis, which reveals only linear epitopes, showed a systematically positive reaction with swine serum at only two time-points after immunization (days 10 and 70) (Fig. 4A). This pattern was different from that seen with ELISA analysis, which detects mainly conformational epitopes (Fig. 4B).

3.4. Cap protein expression in yeast induces spontaneous virus-like particle formation

To determine whether PCV2 Cap protein can form VLPs in yeast, we used electron microscopy to search for nanoparticles in yeast-expressing *opt-cap* gene extracts purified by sucrose gradient centrifugation. Fig. 5A shows that purified yeast extracts contained numerous particles with a size and morphology consistent with that of PCV2 VLPs produced in a baculovirus/sf9 insect cell system. High-resolution microscopy studies showed that these nanoparticles had diameters ranging from 14.9 to 30 nm (mean 25.9 nm), and an icosahedral-like structure that resembled native PCV2 virions (Fig. 5B). These structures were not detected in purified cell extracts made from *S. cerevisiae* wild-type strains (data not shown).

3.5. S. cerevisiae/pYES::opt-cap extracts induce immunogenicity in mice following oral administration

As baculovirus/sf9 produced PCV2 VLPs are structurally and antigenically indistinguishable from regular PCV2 capsids, and can induce immune responses against native virions [20], we examined whether VLPs derived from *S. cerevisiae*/pYES::*opt-cap-* could induce specific antibodies against baculovirus/sf9-produced PCV2 VLPs. Serum and fecal PCV2 Cap antibody levels in mice were measured following oral-gavage of raw extracts of recombinant yeast containing approximately 100 μg of Cap protein. Significant levels of serum IgG and IgM (Fig. 6A) and fecal IgA (Fig. 6B) against baculovirus/sf9-produced PCV2 VLPs were detected in mice 15 days after the second administration. The levels of both serum and fecal antibodies increased significantly after further immunizations and reached peak levels following the fifth and third immunization, respectively. In contrast, PCV2 antibodies were barely detectable in mice immunized with *S. cerevisiae*/pYES2 or in the unvaccinated control group, suggesting that immunity against PCV2 is specific and is elicited by VLPs derived from *S. cerevisiae*/pYES2::*opt-cap*. All orally vaccinated mice remained healthy and none of them exhibited noticeable side effects during the course of the study (data not shown).

4. Discussion

The goal of the present study was to establish a basis for an oral yeast-based vaccine against PCV2 using mice as a mammalian model. The serological and mucosal antibody response of mice fed raw *S. cerevisiae* extracts expressing PCV2 Cap was examined. This protein was chosen because it is the most immunogenic PCV2 protein available and because it is responsible for the induction of PCV2-neutralizing antibodies [48]. The recombinant model resulted in the formation of virus-like particles of PCV2, and because it is simple to scale-up, this system has potential for the implementation of a production-scale vaccination program.

The first experiments using transformants with native PCV2 sequence gave a low transformation yield and almost undetectable PCV2-Cap expression levels (data not shown). This has been attributed to protein toxicity, lack of availability of the proper aminoacyl-tRNAs (codon usage), or DNA-associated toxicity [49]. To improve the expression levels of recombinant *S. cerevisiae,* a yeast codon-optimized PCV2 *cap* gene (*opt-cap*) was chemically synthesized. Yeast transformed with the PCV2-*opt-cap* gene had a higher transformation efficiency and showed significant expression levels of Cap protein (Fig. 2), suggesting that the protein is not toxic in yeast and that, at first glance, the *cap*-derived codon usage affects negatively yeast metabolism. However, the possibility that the secondary structure of PCV2 *cap* DNA is intrinsically toxic to yeast cannot be ruled out, since codon optimization led to a global change in the DNA sequence (Fig. 1) and resulted in a different secondary structure for the *cap* DNA coding sequence (unpublished results).

S. cerevisiae transformed with the yeast-optimized PCV2 *cap* gene expressed an abundant protein consistent with the Cap protein (Fig. 2). The protein was recognized by both anti-PCV2 specific antibodies (Fig. 3) and by sera obtained from either PCV2-vaccinated (Fig. 4) or PMWS-affected pigs, suggesting that the antigenicity of PCV2 protein remained after optimization and heterologous expression in yeast. The yeast-produced Cap protein had the same electrophoretic mobility as *E. coli*-produced 6xhis-Cap fusion protein. However, a shift in mobility was seen by blotting and SDS-PAGE analysis of Cap protein produced in a baculovirus/sf9 cell system (Fig. 3). This shift in protein mobility probably resulted from host-specific and differential post-translational chemical modifications. Although the PCV2 *cap* gene is predicted to contain only one glycosylation site [2], it is possible that it may encode proteins with other sites for alternative post-translation modification that might account for the different sizes of the proteins produced in different hosts [4].

Yeast-produced Cap protein also self-assembles into VLPs that are consistent with PCV2 in size and shape (Fig. 5). However, some of the yeast-produced VLPs observed were less homogenous in size and shape than reported for the native PCV2 virion. A similar observation was made by the authors of the first report for recombinant-PCV2 Cap protein expressed in a baculovirus/insect cell system, suggesting that these differences may be due to the absence of minor structural proteins or slightly impaired DNA regulation [50].

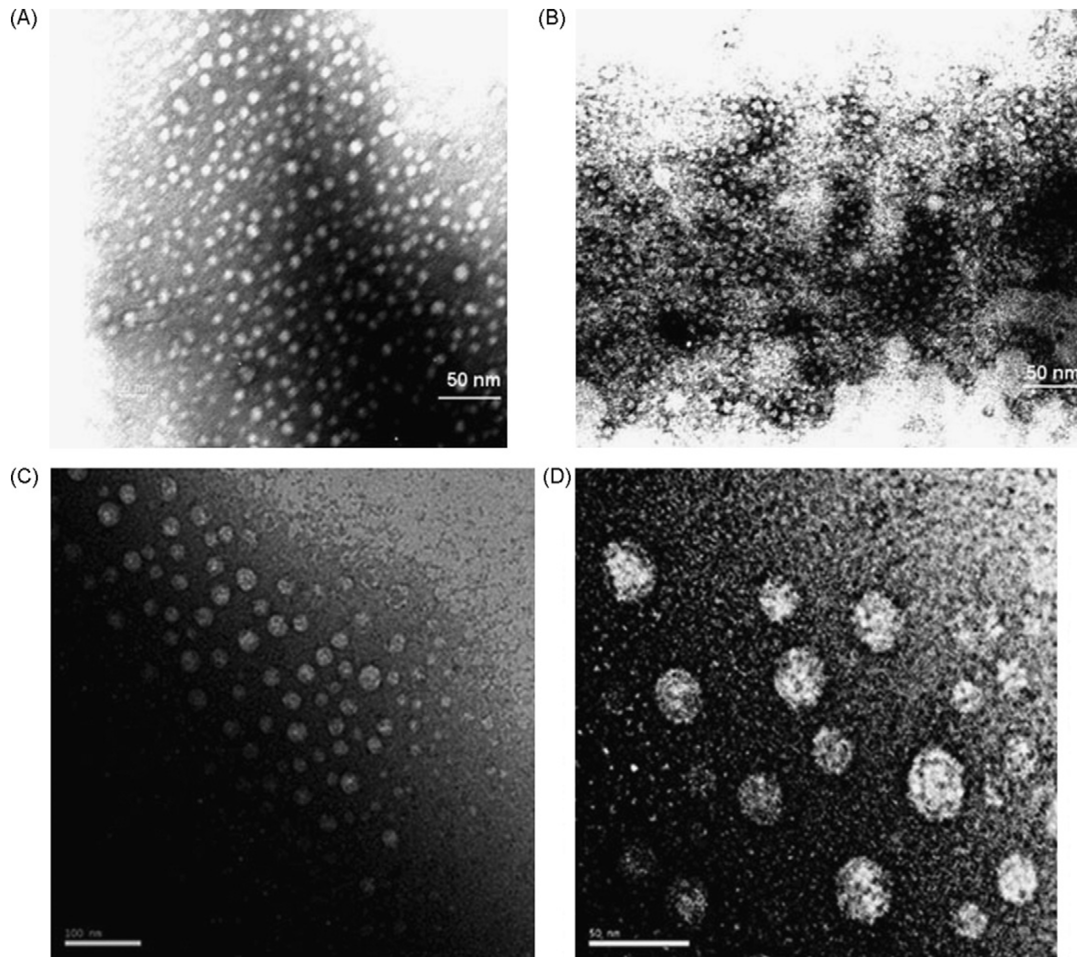

Fig. 5. Electron micrographs of PCV2 Cap protein preparations. Purified recombinant Cap protein expressed in insect cells (panel A) and purified recombinant Cap protein expressed in yeast (panel B) were viewed using a TEM Zeiss EM 109–80 kV. Magnification is 85,000×. Panels C and D, high-resolution transmission electron micrographs (HR-TEM) of purified recombinant Cap protein expressed in yeast viewed using a FEI Tecnai F20 FEG HR-TEM operating at 200 kV. Magnification is 21,000× for panel C and 71,000× for panel D.

In this study, a mice model was used to evaluate *opt-cap*-recombinant yeast as a potential PCV2 vaccine. Although there is no evidence for natural PCV2 infection in mice, and the clinical signs of intraperitoneally infected animals are different from those displayed by pigs with naturally occurring PMWS [51], this model was chosen because PCV2 can infect and replicate in mice [52], and because previous PCV2 vaccines tested in mice have been shown to provide protection in swine [37].

Mice repeatedly immunized by oral gavage with extracts of *S. cerevisiae*/pYES2::*opt-cap* generated high levels of fecal and serum anti-PCV2 VLP antibodies, with significantly more of the antibodies belonging to the IgG (H & L specific) and IgA classes than in mice immunized with extracts of *S. cerevisiae*/pYES2, after the second immunization ($P < 0.01$). The levels of both serum and fecal antibodies increased concomitantly, and this became more apparent after subsequent immunizations. The significant highest levels were reached following the fifth immunization for serum antibodies ($P < 0.05$) and fallowing the third immunization for fecal antibodies ($P < 0.05$). The amount of specific IgM in the group vaccinated with extracts of *S. cerevisiae*/pYES2::*opt-cap* was higher than in the *S. cerevisiae*/pYES2 group, but only in the interval between the third and fourth immunizations ($P < 0.01$) (Fig. 6). Taken together, these results suggest that induction of Cap-specific IgA and IgG/IgM antibodies is dose- and time-dependent and is increased by oral immunization with raw extracts of *S. cerevisiae* expressing PCV2 Cap protein without additional adjuvant. A significant antibody response can be induced by repeated immunization with a low dosage (approximately 100 μg) of Cap protein in mice.

In this study, achieving successful initiation of mucosal immunogenicity depended not only on effective antigen delivery to the mucosal target, but also on three additional factors. First among these was the natural folding and self-assembly into VLPs of yeast-produced Cap protein. This process enhances recognition of the antigens by gut-associated lymphoid tissues, and primes mucosal, humoral, and probably cell-mediated immune responses. Furthermore, it is thought that this process contributes to the adjuvant effect by enhancing uptake of VLPs into antigen presenting cells (APCs) [53]. Second among these was the particulate nature of the yeast-delivered antigen, which enables specialized M cells to transfer antigenic material to APCs more efficiently than non-particulate antigens [39,54]. The third factor was the adjuvant effect of yeast cell wall constituents. Previous reports have shown that yeast cell wall components, including β-glucan, are able to stimulate both antigen-specific and non-specific immune responses [32,40,55].

IgA and IgG have been shown to play important, multiple roles in animal protection against viral diseases, such as preventing viral adherence, and to have additional roles in immune exclusion and clearance [39,56]. However, the important role of cell-mediated immunity in viral infections is worthy of further experimental investigation.

These preliminary results suggest that oral yeast-mediated PCV2 VLP delivery could be utilized as an alternative strategy to develop

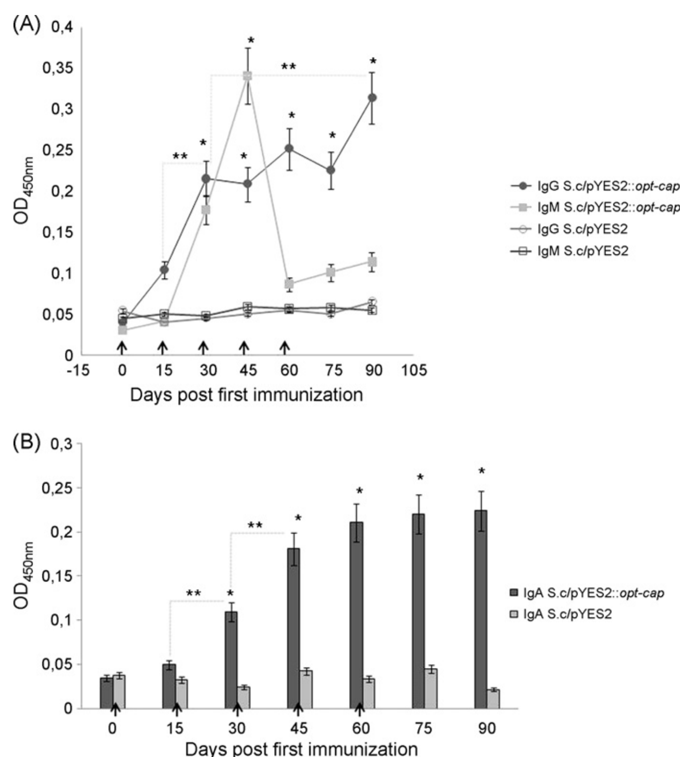

Fig. 6. Serum and fecal Cap-specific antibody profiles of mice immunized orally with control *S. cerevisiae*/pYES2 extracts or recombinant *S. cerevisiae*/pYES2::*opt-cap* extracts. Mice were immunized by oral gavage with the indicated extracts at 0, 15, 30, 45, and 60 days. Individual serum (panel A) and fecal (panel B) samples were collected at 15, 30, 45, 60, 75, and 90 days after initial immunization and subjected to ELISA IgG/IgM and IgA analysis, respectively. The ELISA results are expressed as $OD_{450\,nm}$ values. Data were recorded as the mean and standard deviation of five mice per treatment group. Mean values were significantly different between the experimental groups ($^{*}P < 0.01$, Tukey's post-test) and over time ($^{**}P < 0.05$, Tukey's post-test). Arrows show the immunization schedule.

a new generation of vaccines against PCV2 infection and that this system is worthy of further investigation in the pig, the natural host for PCV2. Several caveats remain to be addressed, including the optimal protective feeding dose for yeast extract in swine, feasibility and cost effective analysis, as well as the effects of adding protective agents to the formula, such as PLGA [57] or other elements that extend particulate antigen persistence in the swine jejunum and terminal ileum [58], the main absorption regions for oral vaccines.

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