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Immunogenicity and immunoprotection of porcine circovirus type 2 (PCV2) Cap protein displayed by *Lactococcus lactis*

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ABSTRACT

The capsid (Cap) protein, an important immunoprotective protein of porcine circovirus type 2 (PCV2), was expressed on the cell surface of the Gram-positive food-grade bacterium, *Lactococcus lactis*. Cap protein was fused to the peptidoglycan binding domain (known as the protein anchor domain, PA) of the lactococcal AcmA cell-wall hydrolase. The Cap protein fusion was non-covalently rebound to the surface of non-genetically modified, non-living high-binder *L. lactis* cells (designated Gram-positive enhancer matrix (GEM) particles). Expression of the recombinant GEM-displaying capsid protein (GEM-PA-Cap) was verified by Western blotting and immunofluorescence and transmission electron microscopy assays. To evaluate the immunogenicity of the recombinant Cap protein (rCap), 20 PCV2-seronegative piglets were immunized with the GEM-PA-Cap subunit vaccine, GEM alone, or phosphate-buffered saline (PBS, challenge control and empty control). Each group consisted of five piglets. The results showed that the level of PCV2-specific antibodies in piglets immunized with the GEM-PA-Cap subunit vaccine was significantly higher than that of the piglets immunized with GEM alone or the control group at all the time points post-vaccination ($P < 0.01$). After challenge with the PCV2 wild-type strain, piglets that received the GEM-PA-Cap subunit vaccine showed significantly higher average daily weight gain (DWG) and shorter fever duration than the other two groups ($P < 0.001$). Furthermore, a significant reduction in the gross lung lesion scores and lymph node lesion scores was noted in the GEM-PA-Cap-immunized group compared with the scores of the GEM or PBS-treated group ($P < 0.01$). The results suggest that recombinant rCap displayed by *L. lactis* GEM particles provided the piglets with significant immunoprotection from PCV2-associated disease. Thus, the novel GEM-PA-Cap subunit vaccine has potential to be considered an effective and safe candidate vaccine against PCV2 infection in piglets.

1. Introduction

Porcine circoviruses (PCVs), including PCV1 and PCV2, belong to the genus Circovirus within the Circoviridae family [1]. PCV1 is nonpathogenic [2] whereas PCV2 is associated with several diseases collectively named porcine circovirus-associated disease (PCVAD) [3–6]. The PCVAD umbrella includes diseases such as post-weaning multisystemic wasting syndrome (PMWS), which is characterized by reproductive failure, enteritis, respiratory disease, and porcine dermatitis and nephropathy syndrome [7]. PCV2 infection is widespread in almost all pig-producing countries and is recognized, therefore, as a global disease in swine [2,8].

Currently, control of PCV2 infection in piglets is based on vaccines. These vaccines are mainly inactivated viral vaccines (e.g., Circovac®, Merial and Fostera™, PCV Pfizer Animal Health, Inc.), or recombinant protein-based vaccines (e.g., Ingelvac CircoFLEX®, Boehringer Ingelheim; Circumvent®, Intervet/Merck and Porcilis® PCV, Schering-Plough/Merck) [9]. All such vaccines have been shown to be effective in reducing PCV2 infection, but fail to prevent infection and spread of PCV2 virus in pigs [8]. In addition, the relatively low viral titers produced by the PCV2-infected PK15 cell line result in a high production cost for the killed viral vaccine [10]. Furthermore, genetically modified recombinant protein-based vaccines pose a potential biological risk [11]. Therefore, one of the major challenges in the control of PCV2 infection is the development of safe, effective and low-budget PCV2 vaccines.

The ability to display heterologous proteins or peptides on the surface of bacteria is important for progress to be made in

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several areas of biotechnology, including the development of vaccine delivery systems [12]. A novel method for surface display of an antigen on genetically unmodified Gram-positive bacteria has been successfully developed through use of a protein anchor (PA) derived from the *Lactococcus lactis* peptidoglycan hydrolase, AcmA [13–18]. For safety, this system is based on non-living and non-genetically modified Gram-positive bacterial cells, designated Gram-positive enhancer matrix (GEM) particles. The particles are used as substrates that bind to externally added heterologous antigens by means of a high-affinity PA. The GEM-PA surface display system eliminates the risk of undesirable shedding of recombinant DNA into the environment with the attendant genetic modification transfer risk to other organisms. Additionally, the GEM-PA surface display system has potential use for antigen purification, which offers the important advantage of cutting production costs.

ORF2 encodes capsid (Cap) protein, a major immunogenic protein responsible for the production of PCV2-specific neutralizing antibodies [19,20]. Cap protein, with its highly conserved epitopes and ability to induce a strong immune reaction against sera from PCV2-positive animals, has been employed for development of vaccines and serodiagnostic assays [20,21]. Use of the GEM-PA surface display system has been well studied [13–18]; however, development of GEM-PA-Cap as a vector for PCV2 vaccines has yet to be explored.

Consequently, we report here on the immunogenicity of the PCV2 subunit vaccine with the PCV2 Cap protein displayed by the GEM-PA system. We also evaluated the ability of this subunit vaccine to induce humoral immune response and protective immunity against PCV2 in piglets.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. lactis MG1363 was purchased from the China Committee for Culture Collection of Microorganisms (Beijing, China) and was grown in M17 medium containing 1% glucose at 30 °C in standing cultures. *Escherichia coli* DH5α and BL21 (DE3) were purchased from TaKaRa (Dalian, China). *E. coli* DH5α and BL21 (DE3), used as the cloning hosts, were grown in Luria-Bertani (LB) medium at 37 °C or on LB medium solidified with 1.5% (wt/vol) agar. For plasmid selection, 100 mg/L of ampicillin (Sigma, USA) was added.

2.2. PCR amplification of the pa gene

L. lactis MG1363 was grown in M17 medium containing 1% glucose at 30 °C for 18 h in standing cultures. Bacteria were collected by centrifugation (6000 × *g* for 5 min at room temperature) and washed twice with sterile phosphate-buffered saline (PBS, pH 7.4). Total RNA isolated from *L. lactis* MG1363 according to the manufacturer's protocol (TaKaRa) was treated with the RNase-free DNase I (TaKaRa) to remove genomic DNA contamination. First-strand cDNA was synthesized from total RNA with the PrimeScript™ RT-PCR Kit (TaKaRa) and stored at −80 °C.

PCR reactions were performed to amplify PA cDNA. PCR primers were designed based on the sequence in the *L. lactis* cell-wall hydrolase Acm A cDNA library (GenBank ID: U17696.1, corresponding to nucleotides 904–1488). The primer sequences 5′-*GGATCC*ACTACTTATACCGTCAAATC-3′ (sense) and 5′-*CTCGAG*TTTTATTCGTAGATACTGAC-3′ (antisense) were synthesized commercially by Sangon Biotech (Shanghai) Co., Ltd., China. *Bam*HI and *Xho*I restriction enzyme sites in the primers are italicized. The PCR reaction conditions were as follows: initial denaturation at 95 °C for 5 min, 30 cycles at 94 °C for 45 s, 54 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. PCR products, confirmed by visualization after electrophoresis on 1% agarose gels, were cloned into the pMD18-T Simple Vector (TaKaRa) for sequence confirmation.

2.3. Synthesis of the cap gene

A codon-optimized *cap* gene (PCV2 ORF2, GenBank ID: KC153106.1) was synthesized (Invitrogen, Shanghai, China) for heterologous expression in *E. coli*. One of the characteristics of the PCV2 *cap* gene, the region encoding the N-terminal 41 amino acid residues, was deleted, while *Nde*I and *Bam*HI restriction sites were added to the 5′- and 3′-end of the gene.

2.4. Construction of the pa-cap expression vector and transformation of E. coli cells

Restriction enzymes, T4 DNA ligase, and Taq DNA polymerase were obtained from TaKaRa and used according to the manufacturer's instructions. The *pa* and *cap* genes were cloned together as *Nde*I–*Bam*HI and *Bam*HI–*Xho*I restriction fragments into the *Nde*I–*Xho*I-restricted integration vector pET-32a, resulting in pET-32a-*pa-cap* (Supplementary Fig. S1). The pET-32a-*pa-cap* vector was isolated, confirmed as containing the correct fragments by double-enzyme cleavage, and maintained in *E. coli* DH5α. The pET-32a-*pa-cap* and pET-32a (negative control) plasmids were transformed into *E. coli* BL21 (DE3), from which the BL21 pET-32a-*pa-cap* and BL21 pET-32a strains were obtained.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.09.007.

2.5. Expression of the PA-Cap fusion protein

A single colony from the recombinant expression strains was grown in LB medium containing 100 μg/ml of ampicillin at 37 °C until the optical density at 600 nm reached 0.6. Next, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. The culture was incubated for an additional 24 h at 16 °C and then harvested by centrifugation at 6000 × *g* for 5 min at 4 °C, washed twice in distilled water and resuspended in PBS (pH 7.2). Cells were broken by sonication and centrifuged at 12,000 × *g* for 10 min at 4 °C, after which the proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis. The intensity of the PA-Cap bands was compared with a bovine serum albumin (BSA) standard also analyzed by SDS–PAGE.

2.6. Western blot analysis

Expressed proteins were resuspended in equal volumes of 2× SDS–PAGE sample buffer (125 mM Tris–HCl, pH 6.8; 20% glycerol; 4% SDS; 0.25% bromophenol blue) in the presence or absence of 10% β-mercaptoethanol. Proteins were separated by 12% SDS–PAGE and then transferred by electroblotting onto PVDF transfer membranes (Millipore, USA) using a semidry transfer cell (Bio-Rad) according to the manufacturer's manual. Membranes were blocked with 5% BSA in TBST (10 mM Tris/HCl, pH 7.5; 150 mM NaCl; 0.1% Tween 20). The membrane was incubated first with a 1:200 dilution of a porcine antiserum specific for PCV2 (VMRD, Pullman, WA, USA) overnight at 4 °C. After washing, the membrane was incubated with anti-porcine IgG goat antibody (1:10,000) conjugated to peroxidase at room temperature for 1 h. The washing procedure was repeated, and the reactions were visualized with metal-enhanced diaminobenzidine (DAB).