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High-level expression and immunogenicity of porcine circovirus type 2b capsid protein without nuclear localization signal expressed in *Hansenula polymorpha*

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ABSTRACT

Currently, porcine circovirus type 2b (PCV2b) is the dominant PCV2 genotype causing postweaning multisystemic wasting disease (PMWS) in pigs worldwide. Efforts have been made to develop various recombinant capsid proteins of PCV2b used in vaccines against PCV2b. However, the nuclear localization signal (NLS) of PCV2b capsid protein (CP) was found to inhibit the expression of the whole length capsid protein in *E.coli*. Here, we expressed a NLS-deleted capsid protein (ΔCP) of PCV2b in *Hansenula polymorpha* based on the capsid protein of PCV2b strain Y-7 isolated in China. Comparatively, the ΔCP was expressed at a higher level than the CP. The purified ΔCP could self-assemble into virus like particles (VLPs) with similar morphology of the VLPs formed by CP. The purified ΔCP could be recognized by the anti-sera derived from the mice immunized by inactivated PCV2b particles. Furthermore, it induced higher levels of PCV2b specific antibodies than the purified CP in mice. These results showed that the ΔCP, a recombinant PCV2b capsid protein without nuclear localization signal sequence, could be efficiently expressed in *Hansenula polymorpha*, and used as a candidate antigen for the development of PCV2b vaccines.

1. Introduction

Porcine circovirus type 2b (PCV2b), one of the major PCV2 epidemic genotypes, encompasses small single-stranded circular genome and nonenveloped DNA viruses and belongs to the family Circoviridae [1,2]. This virus is identified as the virulent porcine pathogen causing postweaning multisystemic wasting disease (PMWS) [3–5]. After the first confirmed case in Canada in 1997, PMWS was subsequently identified in pigs in other countries such as USA and China [6,7]. The disease causes illness mainly in 5- to 12-week-old piglets with clinical signs including dyspnea, jaundice, weight loss and enlarged lymph nodes, lymphocytic depletion, multinucleated giant cell formation, multifocal lymphohistiocytic pneumonia, as well as degeneration and necrosis of hepatocytes [4]. These signs result in PMWS being a major economic concern in all pig-producing areas of the world. Vaccines can play a major role in preventing PMWS. Commercial PCV2 vaccines have been reported to reduce mortality rates and significantly decrease PCV2 viremia in PMWS affected pigs [8–10]. However, presently, all commercial vaccines are produced on the basis of PCV2a which was another dominant epidemiological PCV2 genotype before 2005. PCV2b has become the more dominant genotype in recent years by studies [11–13], and a 2b-based vaccine has shown better efficacy than a 2a-based vaccine to protect against PCV2 in pigs [14], indicating that it is advisable to produce a 2b-based vaccine to protect the pigs against the new genotype.

To develop the PCV2 vaccines, PCV2 capsid protein, an immunogenic protein encoded by the ORF2 in the PCV2 genome containing the major carrier of type-specific epitopes, was expressed in multiple in vitro expression system [15,16]. The three most frequently used baculovirus-based PCV2a subunit vaccines worked successfully [14,17,18]; However, these vaccines are relatively expensive. Although a bacterial expression system is simple to perform and is commonly used to produce PCV2 vaccines [19], it lacks the ability of protein post-translation modification. Moreover, the capsid protein of PCV2 was reported with a glycosylation site in the 143/145 site [20]. Protein folding and post-translational modification plays important role in the

Abbreviations: *H.polymorpha*, *Hansenula polymorpha*; PCV2b, Porcine circovirus type 2b; NLS, Nuclear localization signal; CP, Capsid protein; VLP, Virus like particle

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effect of the immune response. Yeast, as the primary eukaryotic organism and owning the ability of post-translational modification like other higher organisms, has been successfully used to produce the HPV VLP vaccine [21] and HBV VLP vaccine [22]. In recent years, the advantage of yeast expression systems for developing a PCV2 vaccine has been recognized by several authors. For a preliminary trial of oral vaccine, *saccharomyces cerevisiae* was used as a PCV2b antigen deliver [23]. High-level and secretory expression of PCV2b capsid protein in *Pichia pastoris* was also reported [24]. However, there exist different viewpoints about the influence of nuclear localization signal (NLS) at the N-terminus of PCV2b ORF2 for PCV2b capsid protein secretory expression in *Pichia pastoris*.

It was reported that the NLS of PCV2 capsid protein consists of 41 amino acid residues at N-terminus and is responsible for the strict nuclear targeting of PCV2 capsid protein [25]. The NLS of PCV2 capsid protein can influence its expression in *E.coli*. The codon-optimized capsid protein without NLS was expressed in both BL21-CodonPlus (DE3)-RIPL cells and BL21 (DE3) AI cells, while the whole codon-optimized capsid protein failed to expression in BL21 (DE3) AI cells [26]. Similar to this result, the recombinant NLS-defected capsid protein of PCV2 fused to glutathione S-transferase expressed in *E.coli* BL21 strain successfully, while capsid protein with NLS failed to express in this expression system [27]. In addition, it was demonstrated that the first 47 amino acid residues at N terminus of PCV2 capsid protein showed poor reactivity with positive swine sera in an indirect ELISA experiment [28] and were not involved in the formation of conformational epitopes [29]. However, high-level expression of the capsid protein with PCV2b-mimic immunogenicity is a prerequisite for the PCV2b vaccine development.

In the present study, in order to raise the expression level and immunogenicity of PCV2b capsid protein, we expressed and purified the codon-optimized and NLS-deleted PCV2b capsid protein in *Hansenula polymorpha*. The data showed that the NLS-deleted capsid protein of PCV2b could be expressed at a high-level and induce specific antibody against PCV2b in mice, indicating that it could be used as a candidate antigen in PCV2 vaccines.

2. Materials and methods

2.1. Construction of recombinant expression plasmids

Based on the porcine circovirus 2 strain Y-7 amino acid sequence deposited in Gene bank (NO: KF027497.1), the PCR primers were designed and synthesized for amplification of the wild-type coding region of PCV2b capsid protein (wt-CP). The codon-optimized capsid protein (CP) of PCV2b was synthesized by 9 round-overlapping PCR. The first round of amplification was carried out for 30 cycles. The temperature profile for first round was 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min. The PCR product verified on a 2% agarose gel electrophoresis was diluted and used as a template for the next round PCR. The overlapping PCR was carried out for nine rounds. The gene of NLS-deleted PCV2b capsid protein (ΔCP) was amplified by PCR using the CP gene as a template and the primers designed according to the gene sequence of the 42-234 amino acids residues of codon optimized CP. 6 × His Tag encoding gene was integrated into the downstream of the CP encoding gene and ΔCP encoding gene. All the amplified products were cloned into the pMD18T-Simple Vector (TaKaRa), and subcloned into the BgL II and Hind III sites of pX shuttle vector designed by our own laboratory. The recombinant plasmids were transformed into *E.coli* using traditional chemical transformation methods and selected on LB agar plates with 50 μg ampicillin/ml. The recombinant plasmids pX/wt-CP, pX/CP and pX/ΔCP were identified by restriction analysis and sequencing, then linearized with Sac I for their transformation in *H.polymorpha*. All the restriction enzymes were purchased from TaKaRa. All the primers in this project were list on Table 1.

2.2. H.polymorpha transformation and expression

About 10 μg Sac I-linearized plasmids were transformed into the Ura3$^-$ *H.polymorpha* host strain by the method using a 2 mm cuvette after an electric field pulse: 2.5 kV, 25 μF, and 200 Ω (resulting the pulse length of ± 5 ms) with Bio-Red Gene Pulser system. The transformants were spread onto the selective autotrophic YNB Ura agar medium for 3 days–6 days at 37 °C. The recombinant colonies were picked and transferred into 5 ml YPD medium, then cultured at 220 rpm/37 °C for 24 h. The positive strains were tested by PCR using aforementioned primers (PAF1/PBR4) and incubated in 5 ml YPG (1% w/v yeast extract, 2% w/v peptone, 1% v/v glycerol) at 220 rpm/37 °C until they reached a density of 2–6 at OD_{600}. The cells were harvested and, after being washed twice with PBS, inoculated into induction YPM medium with appropriate volume for the density about 1 at OD_{600}. During the subsequent 24 h (until 72 h) methanol was added to achieve a final concentration of 1%. The cells were incubated at 37 °C with shaking, and were harvested by centrifugation at 1500 × g for 5 min at 4 °C prepared for the further SDS-PAGE and Western blot analysis.

The expressed yeast cell lysate mixed with SDS-PAGE loading buffer containing β-Mercaptoethanol, then loaded on 12% SDS-PAGE after heating the samples for 10 min at 100 °C. The polyacrylamide gel was stained using Coomassie Blue for visualizing the protein. Quantitative proteins analysis was performed on a Tanon GIS system (Tanon, China).

2.3. Western blot

The recombinant proteins resolved on SDS-PAGE gels were also transferred to nitrocellulose membrane (Whatman, Germany) using TE 77 PWR Semi-Dry Transfer Unit (GE Healthcare, USA) according to the manufacturer's manual for 1 h. The membrane was blocked with 5% milk for 2 h at room temperature, and incubated at room temperature for 2 h with anti-his tag (ProteinTech Group, Chicago, USA) diluted 1:5000 in 5% milk or antiserum against inactivated PCV2b diluted 1:200 in 5% milk. After being washed with PBST (50 mM potassium phosphate, 150 mM NaCl, 0.05% Tween 20, pH 7.2) for three times at interval 5 min, the membrane was incubated with a goat anti-mouse IgG (H + L)-horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories, USA) for 2 h at room temperature. After further washing, the signal was detected using the colorimetric substrate DAB/H_2O_2 in the dark place for color development. *Blue Plus*® Protein Marker (14 kDa - 100 kDa, TransGen Biotech) was used on Western blot membranes.

2.4. Analysis of copy number by quantitative real-time PCR

Genome DNA from yeasts grown in YPD was prepared by using the disruption buffer (200 mM AcLi, 500 mM NaCl, 10 mM EDTA, 100 mM Tris-HCl pH 8.0, 1% SDS) and the isopropyl alcohol organic reagent. Quantitative real-time PCR was performed following the $2^{-\Delta\Delta CT}$ method [30] using a TransStart® Top Green qPCR SuperMix with StepOne™ System (ABI). To determine the copy number of the recombinant gene, untransformed *H. polymorpha*, ideally with one copy of the MOX promoter was used as the calibrator strain [31].

2.5. Purification of the recombinant PCV2b capsid protein

About 4.7 g of wet induced cells of the recombinant *H.polymorpha* expressing ΔCP or CP were harvested and resuspended in 60 ml harvest buffer (50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 500 mM NaCl, 0.03% Tween 80, 1 mM EDTA, pH 6.5). The cell paste lysed over fifteen passages through the cell disrupter at 1000 bar pressure. Cell-lysate was clarified by centrifuging at 6,000 g for 10 min to remove the cell debris. Size-exclusion chromatography was performed in a 2.5 cm × 100 cm Glass Econo-column (Bio-Rad Laboratories Inc., USA) loaded with 377 ml of Sephacryl S-1000 FF (Amersham Pharmacia, Sweden). The