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Short communication

Production of *Escherichia coli*-based virus-like particle vaccine against porcine circovirus type 2 challenge in piglets: Structure characterization and protective efficacy validation

Xiangfeng Xi[a,1], Xiaobing Mo[c,d,1], Yan Xiao[a], Bo Yin[c], Chaochao Lv[a], Yuzhou Wang[a], Zhe Sun[a], Qingyuan Yang[a], Yali Yao[a], Yajie Xuan[a], Xiangdong Li[a], Y. Adam Yuan[c,d,**], Kegong Tian[a,b,*]

[a] National Research Center for Veterinary Medicine, Road Cuiwei, High-Tech District, Luoyang 471003, PR China
[b] College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou, Henan 450002, PR China
[c] National University of Singapore (Suzhou) Research Institute, 377 Lin Quan Street, Suzhou Industrial Park, Jiangsu 215123, PR China
[d] Department of Biological Sciences and Centre for Bioimaging Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543, Singapore, Singapore

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ABSTRACT

We report the strategies leading to the large-production of soluble non-tag full-length porcine circovirus type 2 (PCV2) Cap protein in *Escherichia coli*. Under neutral pH condition, the purified recombinant Cap protein derived from *E. coli* expression self-assembles into homogenous round virus-like particle at the similar size of that of the intact PCV2 virus, which is further characterized by Cryo-EM single particle structure determined at 4.5 Å. The engineered PCV2 rCap VLP was tested as a subunit vaccine for the protective efficacy against PCV2 challenge on 3-week old piglets. Similar to commercial available PCV2 vaccine, the Cap VLP-immunized piglets developed specific antibody-mediated response and were protected from the virulent SH PCV2 strain challenge. Hence, the production of *E. coli* based PCV2Cap-VLP could be applied as a cost-friendly and effective subunit vaccine to control PCV2 spreading in developing countries.

Porcine circoviruses (PCVs) are circular single-stranded DNA viruses, which belong to the genus *Circovirus* in the family of *Circoviridae*. Type 2 PCV (PCV2) was reported to be the causative agent of post-weaning multisystemic wasting syndrome (PMWS), which is present in all major pork producing countries and is considered to be one of the most economically important viral pathogens worldwide (Allan et al., 1998; Gillespie et al., 2009). Commercial PCV2 vaccines include traditional inactivated vaccines such as Circovac® (Merial) and subunit vaccines such as Ingelvac CircoFLEX® (Boehringer Ingelheim) (Beach and Meng, 2012). These subunit vaccines are based on PCV2Cap protein, the product of PCV2 ORF2, expressed in baculovirus system. The recombinant PCV2Cap proteins expressed in insect/baculovirus and yeast systems are self-assembled *in vivo* to form virus-like particles (VLPs), which provide protection against field PCV2 infection (Bucarey et al., 2009; Fan et al., 2007; Nawagitgul et al., 2000). Compared to eukaryotic expression systems, developing subunit vaccine by using *Escherichia coli* expression system turns out to be more attractive due to the low cost and the robust manufacturing protocols. Recently, Wu et al. demonstrated that VLPs derived from PCV2 rCap protein produced in *E. coli* could elicit specific antibody responses and provide protection against PCV2 challenge in SPF piglets (Wu et al., 2012). However, the major barrier for non-tag full length soluble PCV2 rCap protein expression in *E. coli* is PCV2Cap protein harbors the high incidence of arginine residues at its N-terminus, which are coded by rare codons used in *E. coli* expression system (Wu et al., 2012).

To circumvent the barrier, we systematically screened the expression and solubility of dozens of PCV2Cap proteins by the combinations of selections of PCV2 strains and expression vectors, followed by rare codon gene sequence optimization by generating genetic codon sequence which allows the coding of same amino acids by different triplets. The optimized Cap gene sequence from

* Corresponding author at: National Research Center for Veterinary Medicine, Road Cuiwei, High-Tech District, 471003 Luoyang, PR China.
** Corresponding author at: National University of Singapore (Suzhou) Research Institute, 377 Lin Quan Street, Suzhou Industrial Park, Jiangsu 215123, PR China.
E-mail addresses: dbsyya@nusri.cn (Y.A. Yuan), tiankg@263.net (K. Tian).
[1] Equal contribution.

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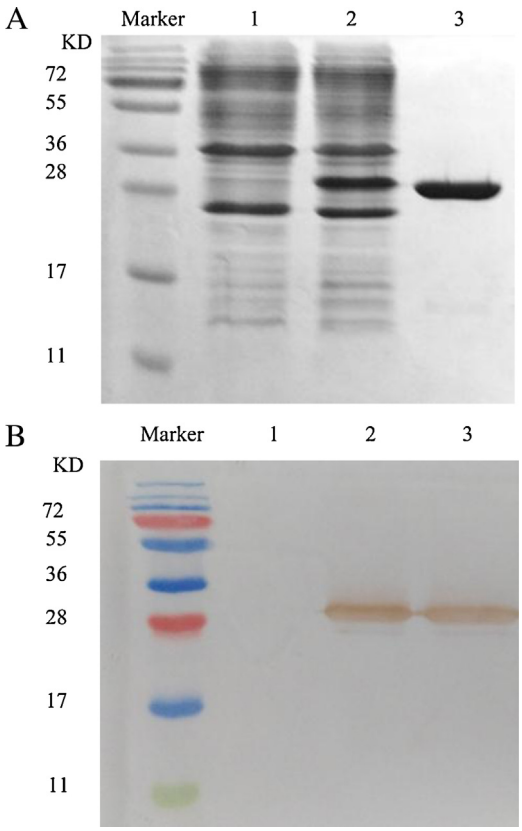

Fig. 1. SDS-PAGE and Western-blot analysis of PCV2 capsid protein. (A) SDS-PAGE analysis. Line1, uninduced bacterial lysates; Line2, IPTG-induced bacterial lysates; Line3, purified rCap protein after ion-exchange chromatography. (B) Western-Blot analysis. Line1, uninduced bacterial lysates; Line2, IPTG-induced bacterial lysates; Line3, purified rCap protein after ion-exchange chromatography.

PCV2 GX strain (Suppl. Fig. 1) was cloned into pET24a vector and the recombinant plasmid was transformed into BL21/DE3 competent cells. To test the feasibility of large-production of PCV2 rCap by industrial setting, a fresh transformed clone was seeded to 5 ml LB media, then propagated to 100 ml, 5 l and 100 l by fermentation. The expressed non-tag full length PCV2 rCap protein was analyzed by SDS-PAGE, followed by western blotting (Fig. 1A and B). The SDS-PAGE and western-blot results showed that a large amount of non-tag full-length Cap recombinant proteins have been successfully expressed in the soluble form (Fig. 1A and B). The supernatant of cell lysates containing recombinant Cap (rCap) protein was precipitated by 60% saturated ammonium sulfate and resuspended, followed by anion ion-exchange chromatographic purification. The purified recombinant PCV2Cap proteins have been completely re-assembled into VLPs in a buffer of 50 mM Tris-HCl and 500 mM NaCl. Dynamic light Scattering (DLS) results further confirmed that ~99% of the purified rCap mono-dispersed with an apparent VLP radius of 8.72 nm (Fig. 2A). Moreover transmission electron microscopic analysis showed that the diameter of rCap VLPs range from 15 to 20 nm which consistent with our DLS results and the previous report (Fig. 2B) (Wu et al., 2012). These data demonstrated that soluble full length non-tag rCap protein could be expressed from *E. coli* and self-assembled into VLPs by combination of PCV2b strain selection and codon optimization.

To compare the structural similarities between our *in vitro* assembled PCV2Cap VLP with the *in vivo* assembled PCV2 N12Cap VLP and validate the presence of the structural epitopes at the surface of the *in vitro* assembled VLP, we determined the Cryo-EM single particle structure of *in vitro* assembled PCV2Cap VLP at 4.5 Å resolution (Fig. 3A and B). The crystal structure of the published truncated PCV2Cap protein with consensus sequence was docked into the calculated VLP density map [20]. Similar to the Cryo-EM structure of *in vivo* assembled PCV2 N12Cap VLP, *in vitro* assembled PCV2Cap VLP displayed distinguished 5-fold, 3-fold and 2-fold axes (Fig. 3C). Moreover, structural docking of N-terminal 40 residues truncated PCV2Cap proteins into the *in vitro* assembled VLP densities demonstrated that N-terminal 40 residues of PCV2Cap proteins are most likely proximal to the icosahedral 5-fold axes (Fig. 3D) and the surface regions harboring the critical structural epitopes (Fig. 3D).

Notably, the extra density left from the docking of PCV2 crystal structure into the density map of the *in vitro* assembled VLP is significant smaller than the extra density left from the docking of PCV2 crystal structure into the density map of the *in vivo* assembled VLP (Fig. 3D, left panel). Such observation strongly suggested that *in vitro* assembled PCV2Cap VLPs derived from *E. coli* expression by disassembly and re-assembly treatment are free of packaged nucleic acids contamination. Hence, *in vitro E. coli* expressed and re-assembled VLPs could be ideal vaccines with less junk nucleic acids contamination, compared to current baculovirus expressed *in vivo* assembled VLPs.

We next tested the protective efficacy of the purified self-assembled Cap VLPs and compared with a commercial vaccine on 3-week old piglets using one-dose schedule, followed by a virulent PCV2b SH strain challenge. Twenty 3-week old PCV2 antigen-free piglets were randomly assigned to four groups with 5 animals in each group. Groups 1 and 2 were vaccinated intramuscularly once with 1 ml experimental subunit vaccine (rCap, 40 μg/pig with Montanide™ Gel 01 adjuvant at 1:9 ratio (v/v)) and one dose of a commercial subunit vaccine (Circoflex, Boehringer Ingelheim Vetmedica Inc., 1 ml/pig as the manufacturer's instruction), respectively. Groups 3 and 4, sham-vaccinated with PBS, were used as