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Porcine parvovirus capsid protein expressed in *Escherichia coli* self-assembles into virus-like particles with high immunogenicity in mice and guinea pigs

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ABSTRACT

Porcine parvovirus (PPV) is a causative agent of reproductive failure in pregnant sows. Classical inactivated vaccine is extensively used to control PPV infection, but problems concerning safety, such as incomplete inactivation may occur. In this study, a novel subunit vaccine against PPV based on virus-like particles (VLPs) formed from the complete PPV VP2 protein expressed in a prokaryotic system with co-expressed chaperones is reported. The VLPs have a similar size, shape, and hemagglutination property to the PPV. Immunization with these VLPs stimulated the neutralization antibody and hemagglutination inhibition (HI) antibody responses in mice and guinea pigs. The lymphocyte proliferation response and cytokine secretion was also induced in immunized guinea pigs comparable to those immunized with PPV inactivated vaccine. In addition, immunization with VLPs also significantly reduced the PPV content in the spleen of guinea pigs 14 days after the challenge with intact virus. These studies suggest that PPV VLPs created as described here could be a potential candidate for vaccine development.

1. Introduction

Porcine parvovirus (PPV) is a causative agent of reproductive failure in pregnant sows that is characterized by stillbirths, mummified fetuses, and early embryonic death (Hueffer and Parrish, 2003; Ren et al., 2013; Krakowka et al., 2000). It is a small icosahedral non-enveloped virus, 25 nm in diameter, and is a member of the *Parvoviridae* family (Cotmore et al., 2014). The genome of PPV is a single-stranded negative-sense DNA, encoding 2 nonstructural proteins and 3 capsid proteins. The capsid proteins VP1 and VP2 are translated by alternate RNA splicing, and VP3 is formed by proteolytic cleavage of the N-terminal of VP2 (Ranz et al., 1989).

The virus particle contains 60 copies of a combination of the 3 structural proteins, and exhibits T = 1 icosahedral symmetry (Simpson et al., 2002). As the major component of the virus, capsid VP2 protein can assemble into virus-like particles (VLPs) alone (Cotmore et al., 2014), it contains most of the B-cell epitopes that play a critical role in eliciting neutralization antibodies. Therefore, VP2 protein is generally considered the major antigen of PPV vaccines (Martinez et al., 1992).

Porcine parvovirus infections are controlled mainly by the humoral response (Nielsen et al., 1991; Cotmore et al., 2014). Although classical vaccines based on inactivated viruses effectively prevents the disease, the potential for incomplete inactivation of the virus can lead to the development of disease (Antonis et al., 2006). Hence, new generation vaccines should be developed. VLP is a kind of subunit vaccine that is composed of viral structural proteins and contains no infectious genetic material, has a similar structure to native virus particles, and is capable of inducing humoral and cellular immune responses (Crisci et al., 2012; Feng et al., 2014). They are recognized efficiently by the immune system and present

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viral antigens in a more authentic conformation than other subunit vaccines (Crisci et al., 2012). To date, VLPs have been produced for about 30 different viruses (Antonis et al., 2006; Maranga et al., 2002). Previous studies have shown that parvovirus-like particles produced from insect cells can be successfully constructed, have excellent immunogenicity, and can protect swine against reproductive failure following virus challenge (Zhao et al., 2014; Noad and Roy, 2003).

At present, several expression systems have been used to produce VLPs, including insects (Zhou et al., 2010), yeast (Guo et al., 2014; Tamosiunas et al., 2014), and bacterial cells (Qi and Cui, 2009), with insect cells the most widely used (Antonis et al., 2006). Foreign proteins expressed in *E. coli* often undergo rapid degradation or aggregation because they are incorrectly folded (Qi and Cui, 2009). Co-expression of molecular chaperones can assist protein folding correctly and so increase the production of active proteins. The most important chaperones in *E. coli* include: GrpE, GroEL, DnaK, DnaJ, and GroES. Several studies have indicated that 2 major chaperone teams, DnaK-DnaJ-GrpE and GroEL-GroES, play an important role in protein folding. The effectiveness of these chaperones on protein folding, stability, and aggregation has been demonstrated (Nishihara et al., 1998).

We report the expression of PPV VP2 protein with a co-expressed GroES-GroEL-tig chaperone team in *E. coli*, and the production of water soluble VP2 protein which assembled into VLPs with a similar size, shape, and hemagglutination property to natural PPV. The animals' immune response induced by PPV VLPs efficiently decreased the infection rate, suggesting that these VLPs could provide the basis of a new vaccine for PPV control.

2. Materials and methods

2.1. Cells and virus

PK-15 cells (ATCC™ CCL-33) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U/ml of penicillin and 100 mg/ml of streptomycin at 37 °C in a 5% CO_2 incubator. PPV reference strain 7909 was propagated in PK-15 cells as previously described (Rueda et al., 2000). Briefly, the virus was inoculated into the monolayer of PK-15 cells of 60% confluency in cell flasks. After incubation at 37 °C for 2 h, the culture medium was discarded and fresh DMEM containing 3% FBS was added, then the cells were cultured at 37 °C continuously for about 48 h. After 80% of the cells showed cytopathic effects (CPE), the cells and medium were collected and freeze-thawed for 3 times. Then the virus was stored at −80 °C until use.

2.2. Plasmid construction

To express the VP2 protein of PPV strain China (GenBank accession number AY583318) in *E coli*, the codon of VP2 gene was optimized, and a new sequence (GenBank accession number KX524499) was formed and synthesized by Sangon, China. The VP2 gene was then amplified by PCR with the primers (Forward: 5′-***CATATG***ATGTCGGAAAATGTGGAACA-3′; Reverse: 5′-***CTCGAG***TTAA-TACAGTTTCCGTGGAATGA-3′). The PCR product was digested with restriction endonucleases *Nde* I and *Xho*I, and subcloned into pET28a vector (pET28a-VP2). After transformation in JM109 competent cells, the plasmid was confirmed by restriction analysis and sequencing.

2.3. Protein expression and purification

Competent cells of *E. coli* BL21 (DE3) harboring chaperone plasmid Tf2 were prepared according to the manufacturer's protocol (TaKaRa, China). Subsequently, plasmid pET28a-VP2 was transformed into the BL21-Tf2 competent cells. A single colony containing both chloramphenicol- and kanamycin-resistance genes was induced by 8 ng/ml of tetracycline and 0.1 mM of isopropyl β-D-thiogalactoside (IPTG) for the expression of the chaperone and VP2 protein, respectively. After induction at 30 °C for 8 h, the cells were harvested, resuspended in buffer A (250 mM NaCl, 50 mM Tris, pH 8.0) and lysed by sonication on ice. After centrifugation at 12,000 g for 20 min, the supernatant was collected and purified by Ni-NTA affinity chromatography. Briefly, the clarified cell lysates were pumped onto buffer A equilibrated Ni-NTA column (Merck, Germany). After washing with 10 beds of buffer B (250 mM NaCl, 50 mM Tris, 30 mM imidazole, pH 8.0), the recombinant VP2 protein was eluted with buffer C (250 mM NaCl, 50 mM Tris, 250 mM imidazole, pH 8.0). Fractions were analyzed by SDS-PAGE and Western blot, and quantified with a Micro BCA™ protein assay kit (Pierce Biotechnology, USA). Finally, the content of bacterial endotoxin in purified VP2 protein was measured by a ToxinSensor™ Endotoxin Detection System (GenScript, USA).

2.4. VLPs assembly and quantification

The purified VP2 was dialyzed against 50 mM of Tris with different concentrations of NaCl (100 mM, 150 mM, 250 mM, 300 mM) and pH (pH 7.0, pH 8.0). The PPV VLPs were detected by transmission electron microscopy (TEM) using the negative staining method and dynamic light scattering (DLS) as described before (Chandramouli et al., 2013). Hemagglutination (HA) assay was performed using mice red blood cells as previously described (Joo et al., 1976).

2.5. Vaccination of mice with PPV VLPs

Twenty female Kunming mice of 3–4 weeks old were divided randomly into 4 groups (n = 5). The mice were inoculated subcutaneously with different doses of VLPs (30 μg, 15 μg), 50 μl of commercial inactivated vaccine (KeQian, China) and 100 μl of PBS, respectively. The VLPs were diluted in 50 μl of PBS and emulsified with 50 μl of Complete Freund's adjuvant, and then 50 μl of Incomplete Freund's adjuvant in the booster immunization. Booster immunization was with the same dose at an interval of 2 weeks. Blood samples were collected from the tail vein each week for 56 days after the primary vaccination.

2.6. Vaccination and challenge in guinea pigs

Thirty six female guinea pigs weighing 400–500 g, were randomly divided into 6 groups: groups A and B were individually inoculated with 100 μg and 50 μg of PPV VLPs mixed with Montandide ISA 71™ VG (Seppic, France) as a W/O emulsion; groups C and D were inoculated with 100 μg and 50 μg of VLPs mixed with $Al(OH)_3$ gel (20 mg/ml, Thermo, USA), group E was inoculated with 100 μl of a standard inactivated PPV vaccine as positive control, and group F was inoculated with 100 μl of PBS as negative control. All of the immunogens were injected into the tibialis cranialis muscle of both rear legs. A booster was given at the same dose at 28 days post primary vaccination, and serum samples were taken from the forelimb veil at 0, 14, 28, and 42 days. At the 42 days after the first immunization, 2 guinea pigs from each group were sacrificed for lymphocyte proliferation assay and cytokine production. The remaining guinea pigs (24) were challenged with 100 μl of $10^{6.5}$ tissue culture infective dose ($TCID_{50}$)/ml PPV 7909 strain as previously described, and monitored for 14 days. The guinea pigs were then sacrificed and the PPV content in the spleen was measured by