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Evaluation of an *omp*A-based phage-mediated DNA vaccine against *Chlamydia abortus* in piglets

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ABSTRACT

Chlamydia abortus (*C. abortus*) is an obligate intracellular pathogen that causes abortion in pigs and poses a zoonotic risk in pregnant women. Although attenuated and inactivated vaccines are available, they do not provide complete protection in animals underlining the need to develop new vaccines. In this study, we tested the hypothesis that intramuscular immunization with an *omp*A-based phage-mediated DNA chlamydial vaccine candidate will induce significant antigen-specific cellular and humoral immune responses. Thus, groups of piglets (five per group) were immunized intramuscularly with the phage-MOMP vaccine (λ-MOMP) or a commercial live-attenuated vaccine (1B vaccine) or a GFP-expressing phage (λ-GFP) or phosphate buffered saline (PBS) (control) and antigen-specific cell-mediated and humoral immune responses were evaluated. By day 63 post-immunization, the λ-MOMP vaccine elicited significantly higher ($P < 0.05$) levels of antigen-specific serum IgG antibody responses than the 1B vaccine or control did. Also, piglets immunized with λ-MOMP vaccine had significantly higher ($P < 0.05$) MOMP-specific lymphocyte proliferative responses compared to those immunized with the 1B vaccine or control. Furthermore, the total T-cell numbers (CD3+) and the proportion of CD4+ and CD8+ T-cell subsets as well as the ratio of CD4+/CD8+ T cells elicited following immunization were comparable between the λ-MOMP- and 1B-vaccinated animals on both days 63 and 70. Interestingly, although the proportion of CD3+CD4−CD8− double negative T cells on day 63 was significantly higher ($P < 0.05$) in the 1B vaccine group compared to the λ-MOMP-immunized group, there was a significant decrease in the proportion of this T-cell population on day 70 in the 1B compared to the λ-MOMP vaccinated group. These results indicate that the λ-MOMP DNA vaccine is capable of inducing antigen-specific cellular and humoral immune responses that may provide protective immunity against a live challenge infection with *C. abortus*.

1. Introduction

Chlamydia abortus (*C. abortus*) is an obligate intracellular bacterial pathogen that undergoes a biphasic developmental cycle characterized by two morphologically distinct forms: an infectious metabolically inert elementary body (EB) and a replicating metabolically active reticulate body. *C. abortus* infection causes abortion and reproductive failure in several animals, including sheep (ovine enzootic abortion), goats and pigs [1,2]. The pathogen is a great threat to human health and brings enormous economic loss to livestock industry [3]. Although antibiotic therapy is effective against *Chlamydia*, it does not always eradicate chronic infection or affect established pathology [3,4]. Additionally, the emergence of multidrug resistant strains has reduced the effectiveness of antibiotics as a successful control strategy [5,6]. Therefore, a reliable and effective vaccine against *C. abortus* is urgently needed for the control of animal Chlamydiosis.

Although a live attenuated vaccine (based on the 1B strain) is currently available for vaccination against *C. abortus* infection in sheep, a recent report confirmed that, in some cases, the 1B vaccine itself caused disease in animals [7]. To develop an effective vaccine, it is important to understand which immunization strategy will elicit a protective immune response in pigs. In previous studies, the 40 kD major outer membrane protein (MOMP) of the *C. abortus* EB was considered to be an important target for generating protective immune responses against *C. abortus* infections [8,9]. Moreover, while DNA vaccines based on the MOMP tend to work well for small animals (e.g., mice), complete protection has not been observed in large animals. This problem could potentially be overcome by the correct choice of delivery system and adjuvant [9,10]. Several reports describing the use of whole bacteriophage particles as vaccine delivery vehicles suggest that this novel delivery method may provide a tool for inducing strong immune responses with DNA vaccines [11,12].

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In this technique, a DNA vaccine expression cassette, consisting of a eukaryotic promoter, a vaccine gene and a polyadenylation site, is cloned into λ-phage, purified whole phage particles and then used to immunize the host. A bacteriophage lambda DNA vaccine expressing the small surface antigen of hepatitis B demonstrated strong antibody responses when compared with a naked DNA vaccine in a rabbit model [13]. Phage vaccines are also particularly attractive due to the fact that they are stable, relatively inexpensive to produce and capable of being delivered orally or intranasally [14]. More recent studies confirmed that immunization with a phage vaccine based on the *omp*A gene of *C. abortus* (λ-MOMP) generated a specific antibody response and produced significant bacterial clearance in mice [15]. In the current study, the *omp*A gene of *C. abortus* was cloned into a standard lambda bacteriophage vector (NM1149) and this phage vaccine was then used to immunize piglets. Immune responses from these piglets were compared to those elicited in piglets immunized with a live attenuated 1B vaccine (Enzovax) [16].We demonstrated that immunization with the λ-MOMP vaccine elicited significantly higher ($P < 0.05$) antigen-specific serum IgG antibody and lymphocyte proliferative responses compared to those immunized with the 1B vaccine or control.

2. Materials and methods

2.1. C. abortus strains

C. abortus strain CP12 (GenBank accession # EF202609) was purchased from the Chinese Institute of Veterinary Drug Control (Beijing, China). The strain was grown in yolk sacs of 7-day-old SPF chicken embryos. Inclusion-forming units (IFU) of *C. abortus* strain CP12 were determined to have a concentration of 1×10^7IFU/ml in McCoy cells. The commercial vaccine strain 1B (Enzovax) (Intervet, Walton manor, Walton) was used as a control.

2.2. Lambda phage vaccine

C. abortus genomic DNA was extracted using the DNAeasy Tissue Kit (Qiagen, Stuttgart, Germany) according to the manufacturer's instructions. The *ompA* gene was obtained by polymerase chain reaction (PCR) amplification from purified genomic DNA based on available primer sequences in GenBank (accession # AJ440239). The expected 1.2 kb PCR product was purified and digested with *Mfel* (a single cut in a non-coding region that yields *Eco*RI compatible ends) and cloned into the *Eco*RI site of purified λNM1149 genomic DNA. λ-MOMP was amplified in *E. coli* strain C600 and then purified and concentrated as described previously [11,14,15]. The bacteriophage vaccine pellet was re-suspended in phage buffer (5.8 g NaCl, 2.0 g $MgSO_4 \cdot 7H_2O$, 50.0 ml 1 M Tris–HCl (pH 7.5), 5.0 ml 2% (w/v) gelatin) and stored at 4 °C before use.

The λ-GFP plasmid was constructed by excising the expression cassette from plasmid pEGFP-C1 and inserting it into the single *Eco*RI site of the NM1149 vector. Phages were grown and purified using standard microbiological techniques and two rounds of CsCl gradient ultracentrifugation were performed as a final purification. Before immunization of piglets, phage stock solution was calculated as plaque-forming unit (pfu) per ml and adjusted to a concentration of 2×10^{12}pfu/ml as previously described [13].

2.3. Immunizations and animal care

All studies were performed on five-week-old weaned piglets (Daxing Pig Farm, Beijing, China). All animals were tested for antibodies against *C. abortus* using an antibody ELISA kit (IDVET, Montpellier, France) before the experiment and animals for positive antibody against *C. abortus* were excluded from the experiment. All procedures were performed in the animal facility of China Agricultural University, Beijing and treated in accordance with the guidelines issued by the Beijing Laboratory Animal Administration Committee on Animal Care.

Twenty piglets were randomly assigned to four groups (five animals per group) and immunized intramuscularly with the vaccines and controls. Piglets in Group 1 received 10^{12} pfu of λ-MOMP phage in 0.5 ml of PBS. Group 2 piglets were immunized intramuscularly with 0.5 ml of 10^{12} pfu of phage λ-GFP and boosted twice as described above. Group 3 piglets were immunized once with 1.5×10^5 IFU of live-attenuated 1B vaccine as a positive control. Group 4 animals were injected intramuscularly with the same volume of PBS as a negative control.

2.4. Humoral response

Blood samples were collected from piglets by ear vein puncture before immunization (day 0) and on days 21, 42, 63 and 84. Serum was separated and stored at −20 °C until processed. Anti-MOMP antibodies in pig serum were detected using a commercial ELISA kit. In addition, anti-GFP antibodies were detected using ELISA plates coated with recombinant GFP protein (Biovision, Milpitas, CA). Briefly, ELISA plates were coated with r-GFP (100 ng/well) in 0.05 M bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. After blocking with 2% BSA/PBS for 2 h at room temperature, the plates were incubated with serially diluted pig sera for 1 h at room temperature. After washing six times with PBS containing 0.5% Tween-20, Horseradish peroxidase-conjugated goat anti-pig IgG antibody (Biosynthesis Biotechnology, Beijing, China) was added to the wells and incubated at room temperature for 1 h. After washing, 3, 3′, 5, 5′-Tetramethyl Benzidine (Sigma, Shanghai, China) substrate was added according to the manufacturer's instructions. The reaction was stopped after 30-min by addition of 2 M sulphuric acid and the optical density was read on a microplate reader at 492 nm.

For detection of anti-phage antibodies, the ELISA plates were coated with 2×10^9 pfu/well of phage antigen in 0.05 M bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C as previously described [12].

2.5. Lymphocyte proliferation assay

Blood samples from each group of animals were collected on day 49. Peripheral blood mononuclear cell (PBMCs) suspensions were prepared, and lymphocyte proliferation was determined using the ^{3}H-thymidine incorporation assay [10,13,17,18]. Briefly, PBMCs were stimulated either with whole phage particles (5 μg/well) or recombinant MOMP protein at 0.2 μg/well or RPMI-1640 medium (negative control). All experiments were performed in triplicate. Plates were incubated at 37 °C in 5% CO_2 for 56 h and then pulsed with 0.25 μCi of [^{3}H] thymidine (Isotope Corporation, Beijing, China) per well for 16 h. The cells were harvested onto glass microfiber filters (Whatman, Kent, UK), and activity was counted in a HIDEX 300SL Automatic TDCR liquid scintillation counter (Nature Gene life Sciences, Beijing, China). Mean values, obtained after subtracting background values, and standard deviations (SDs) were calculated and results were expressed as the stimulation index (SI), calculated as the mean cpm values of stimulated and non-stimulated cells.

2.6. Analysis of T-lymphocyte subsets

PBMCs (2×10^6 cells/ml) isolated from immunized and control piglets were triple-stained with fluorescent-labeled antibodies and used for analysis of T lymphocyte subsets. Briefly, approximately 1×10^6 cells were washed with PBS supplemented with 0.5% fetal bovine serum (M&C Gene Technology, Beijing, China) and pelleted by centrifugation at 500 ×*g* for 2 min. Pelleted cells were re-suspended in 100 μl of a mixture of the mAbs: mouse anti-pig CD4a-FITC, anti-CD8a-RPE and anti-CD3e-SPRD antibodies (Southern Biotech, Birmingham, AL) [18,19] and incubated in the dark at 4 °C for 30 min. Cells were then washed three times with PBS supplemented with fetal