Vaccine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Development of a single-dose recombinant CAMP factor entrapping poly(lactide-co-glycolide) microspheres-based vaccine against *Streptococcus agalactiae*

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ARTICLE INFO

Article history:
Received 18 December 2016
Received in revised form 19 January 2017
Accepted 22 January 2017
Available online xxxx

Keywords:
Streptococcus agalactiae
Vaccine
Mastitis
PLGA
Single dose

ABSTRACT

Streptococcus agalactiae is an important contagious bovine mastitis pathogen. Although it is well controlled and even eradicated in most Northern European and North American dairy herds, the prevalence of this pathogen remains very high in China. However, research on development of a vaccine against *S. agalactiae* mastitis is scarce. The aims of the present study were to: (1) develop a single-dose vaccine against *S. agalactiae* based on poly(lactic-co-glycolic acid) (PLGA) microspheres (MS) encapsulated CAMP factor, a conserved virulent protein encoded by *S. agalactiae*'s *cfb* gene; and (2) evaluate its immunogenicity and protective efficacy in a mouse model. The *cfb* gene was cloned and expressed in a recombinant *Escherichia coli* strain Trans1-T1. The CAMP factor was tested to determine a safe dose range and then encapsulated in MS of PLGA (50:50) to assess its release pattern *in vitro* and immune reaction *in vivo*. Furthermore, a mouse model and a histopathological assay were developed to evaluate bacterial burden and vaccine efficacy. In the low dosage range (<100 μg), CAMP factor had no obvious toxicity in mice. The release pattern *in vitro* was characterized by an initial burst release (44%), followed by a sustained and slower release over 7 wk. In mice immunized with either pure CAMP factor protein or PLGA-CAMP, increased antibody titers were detected in the first 2 wk, whereas only PLGA-CAMP immunization induced a sustained increase of antibody titers. In mice vaccinated with PLGA-CAMP, mortality and bacteria counts were lower (compared to a control group) after *S. agalactiae* challenge. Additionally, no pathological lesions were detected in the vaccinated group. Therefore, PLGA-CAMP conferred protective efficacy against *S. agalactiae* in our mouse model, indicating its potential as a vaccine against *S. agalactiae* mastitis. Furthermore, the slow-release kinetics of PLGA MS warranted optimism for development of a single-dose vaccine.

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1. Introduction

Mastitis is the most common and costly disease of the dairy industry, profoundly reducing bovine milk quality and production in many herds [1]. *Streptococcus agalactiae* is an important contagious mastitis pathogens that is transmitted from cow to cow [2]. This bacterial species is also important from a public health perspective, as it causes sepsis and meningitis in neonates and morbidity in pregnant women and immune-compromised adults [3,4]. However, strains are host-specific [5].

It was traditionally believed that *S. agalactiae* can only survive in the udder because it is an obligate pathogen of the mammary

Abbreviations: BCA, bicinchoninic acid; CAMP, Christie–Atkins–Munch-Petersen; ELISA, enzyme-linked immune-sorbent assay; IMI, intramammary infection; MS, microspheres; PCR, polymerase chain reaction; PLGA, poly(lactic-co-glycolic acid); PLGA-CAMP, PLGA MS encapsulated CAMP factor; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CFU, colony forming units; SPF, specific-pathogen-free; SEM, scanning electron microscopy.

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http://dx.doi.org/10.1016/j.vaccine.2017.01.041
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Please cite this article in press as: Liu G et al. Development of a single-dose recombinant CAMP factor entrapping poly(lactide-co-glycolide) microspheres-based vaccine against *Streptococcus agalactiae*. Vaccine (2017), http://dx.doi.org/10.1016/j.vaccine.2017.01.041

gland and can be eradicated from the herd by implementation of the so-called 5-point mastitis control program [6]. In countries that have adopted this control program, prevalence of *S. agalactiae* intramammary infection (IMI) has decreased dramatically over the past 30 y [7–10]. However, in other countries with emerging dairy industries, herd-level prevalence is still very high (e.g. 60% in Brazil [11] and 92% in China [12]). Recently, prevalence of *S. agalactiae* IMI has increased again in Scandinavian dairy herds, despite implementation of mastitis control programs [13–16]. Therefore, there is an impetus to develop an effective vaccine for mastitis caused by *S. agalactiae*.

Although various approaches have been used to develop vaccines against *S. agalactiae* derived from human sources [3,4], reports on development of a vaccine against *S. agalactiae* as a cause of bovine mastitis are scarce [17]. Inactivated vaccines had no effect on *S. agalactiae* IMI [18,19]. However, X-protein, a surface antigen, was used as a potential immunogen to develop a vaccine, and vaccinated cows developed antibodies that were opsonic to *S. agalactiae* and enhanced its phagocytosis [20]. With rapid developments in genomics, proteomics and *in silico* technologies, a conserved extracellular protein called CAMP factor (23.8 kDa) encoded by the *cfb* gene was identified as a potential antigen against *S. agalactiae* [3,21]. This protein is well known because of the CAMP reaction, which has long been used to identify *S. agalactiae* [22]. The CAMP factor is one of the virulence factors of *S. agalactiae*, damaging susceptible membranes with pore-forming activity [23] and capable of inducing mortality in rabbits and mice [24]. Notably, a CAMP factor-based vaccine induced protective immunity against *Streptococcus uberis* in lactating cows [25], making CAMP factor a potential vaccine candidate for protection against *S. agalactiae*.

Effective delivery and selection of a suitable adjuvant are essential for success of a mastitis vaccine. Poly(lactic-co-glycolic acid) (PLGA), a biocompatible and biodegradable polymer approved by FDA and European Medicines Agency, has been widely used as a carrier to deliver subunit antigens, recombinant proteins, and DNA [26]. Particular advantages of this adjuvant include controlled release kinetics, with potential to develop a single-dose vaccine against *S. agalactiae* [27]. Furthermore, PLGA MS enables pure antigens to induce a more robust immune reaction, as most proteins and peptides have relatively poor intrinsic immunogenicity [28].

The objective of the present study was to assess immunogenicity and protective efficacy conferred by CAMP factor (potential antigen) loaded PLGA MS (PLGA-CAMP) as a one-dose vaccine candidate against *S. agalactiae* in a mouse model. The *cfb* gene from a clinical mastitis *S. agalactiae* strain was cloned and recombinant expression of CAMP factor was established in *E. coli*. The *in vitro* release pattern and *in vivo* humoral immune response were determined, and a mouse model used to evaluate vaccine efficacy.

2. Materials and methods

2.1. Bacterial strains and growth condition

Streptococcus agalactiae BJ-2, isolated from a clinical mastitis milk sample collected from a dairy farm near Beijing, China, was used in this study. A positive CAMP reaction was observed when BJ-2 and *Staphylococcus aureus* (ATCC13565) were cultured on blood agar with 5% sheep blood (SBA). The strain was stored at −80 °C, recovered on SBA and incubated aerobically at 37 °C for 24 h.

2.2. Cloning, expression and purification of cfb gene

Chromosomal DNA of strain BJ-2 was extracted using a genomic DNA extraction kit for bacteria (Sigma-Aldrich, St. Louis, MO, USA). The amplification primer for the *cfb* gene was designed in accordance with published complete sequences (GenBank accession: NC_004116.1), including 5-CGCGGATCCGCGGATCAAGTGACAACT CCA-3, the underlined BamHI site, 5-CCGCTCGAGCGGTTATTTT AATGCTGTTTG-3, and the underlined HindIII site. The polymerase chain reaction (PCR) was performed in a 20 μL final volume containing 1 μL template DNA, 10 μL of Taq plus master mix (Transgen, Beijing, China), 10 pmol of each of the two primers, and RNase-free H_2O. Amplification was done as follows: initial denaturation at 94 °C for 3 min; 30 cycles of 30 s denaturation at 95 °C, 55 s annealing at 55 °C, 1 min extension at 72 °C; and final extension for 7 min at 72 °C. Products of amplification were separated by electrophoresis and confirmed by sequencing. Purified PCR product was ligated with pEASY-T1 Cloning Vector (Transgen). Recombinant pEASY-T1-*cfb* and expression vector pET32a (GE Healthcare, Chicago, IL, USA) were digested by BamHI/HindIII, ligated together, and used to develop recombinant *E. coli* Trans1-T1 (Transgen).

The recombinant *E. coli* strain was cultured at 37 °C for 4 h with agitation (OD 600 nm = 0.6), then 1.2 mM IPTG (Sigma-Aldrich) was added for an additional 4 h for induction. Cells were harvested by centrifugation (15 min, 5000*g*, 4 °C) and resuspended in 0.1 M phosphate buffer (pH 7.4, PBS). Sonication (200 W for 15 min) was performed and CAMP factor was purified from supernatants by affinity chromatography with Glutathione Sepharose 4 Fast Flow resin (Henghuibio, Beijing, China), according to manufacturer's instructions. Detection and removal of LPS were done as described [29]. Protein was quantified with a bicinchoninic acid (BCA) protein assay kit (Solarbio, Beijing, China) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Expression of CAMP factor was confirmed using a Western Blot Kit (Comwin Biotech, Beijing, China) according to the manufacturer's protocol. Anti-His monoclonal antibody (Comwin Biotech) was used as primary antibody and goat-anti-rabbit IgG (H + L)-HRP (Sigma-Aldrich) as secondary antibody.

2.3. Animals and anesthesia

Female 4-wk-old specific-pathogen-free (SPF) BALB/c mice (Merial-Vital Laboratory Animal Technology, Beijing, China) were used. All mice were kept in germ-free isolators and experiments were conducted in compliance with guidelines of the Beijing Municipality on the Review of Welfare and Ethics of Laboratory Animals, approved by the Beijing Municipality Administration Office of Laboratory Animals (BAOLA) and under a protocol (CAU-AEC-2010–0603) approved by the China Agricultural University Animal Ethics Committee. All animals were given 50 mg/kg Zoletil 50 (Virbac, Carros, France) by intramuscular injection before all immunizations, collection of blood samples and bacterial challenge. Mice were euthanized (cervical dislocation) and tissues collected as described [30].

2.4. Safety test of recombinant CAMP factor

To determine safety of recombinant CAMP factor, three groups of BALB/c mice, five in each group, were injected intraperitoneally (ip) with 10 μg, 100 μg or 1 mg of purified recombinant CAMP factor. Negative control mice were given sterile PBS (pH 7.4). All mice were euthanized 9 d post administration and their liver removed and fixed in 10% buffered formalin for histopathological analysis [31].

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