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A recombinant chimera composed of R1 repeat region of *Mycoplasma hyopneumoniae* P97 adhesin with *Escherichia coli* heat-labile enterotoxin B subunit elicits immune response in mice

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Abstract

Swine mycoplasmal pneumonia (SMP), caused by fastidious bacterium *Mycoplasma hyopneumoniae*, is the most important respiratory disease in swine breeding. The commonly used vaccines to control this disease consist of inactivated whole cells (bacterins), whose production cost is high and the efficiency is limited. The objective of this study was to develop and to evaluate in BALB/c mice a recombinant subunit vaccine (rLTBR1) containing the R1 region of P97 adhesin of *M. hyopneumoniae* (R1) fused to the B subunit of the heat-labile enterotoxin of *Escherichia coli* (LTB). rLTBR1 formed functional oligomers that presented high affinity to GM1 ganglioside. Mice inoculated with rLTBR1 by intranasal (IN) or intramuscular (IM) route produced high levels of anti-R1 systemic and mucosal antibodies (IgA), which recognized the native P97. On the other hand, mice inoculated with the inactivated whole cell vaccine did not produce anti-R1 antibodies. The administration route influenced the modulation of the immune response by LTB, showing that IM rLTBR1 induced Th2-biased immune responses and IN rLTBR1 induced Th1-biased immune responses. rLTBR1 administrated by IN route also induced IFN-γ secretion by lymphocytes. rLTBR1 may constitute a new strategy for preventing infection by *M. hyopneumoniae* and may have potential for developing vaccines against other infectious diseases as well.

Keywords: Swine mycoplasmal pneumonia; *Mycoplasma hyopneumoniae*; P97; LTB; Recombinant chimera

1. Introduction

Swine mycoplasmal pneumonia (SMP), caused by fastidious bacterium *Mycoplasma hyopneumoniae*, is present in all countries of the world where a significant swine industry exists. The very high prevalence, coupled with associated losses, makes this respiratory disease the most important in swine breeding. Pigs with primary mycoplasmal infection are pre-disposed to potentially fatal secondary invaders such as *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*, resulting in poor food conversion, retarded growth and even higher mortality [1].

Vaccination appears to be a more effective means of either eradicating or controlling the infection than antibiotics [2,3]. The commonly used vaccines to SMP control consist of inactivated whole cells (bacterins). However, growth of *M. hyopneumoniae* in vitro requires a rich medium, and it is a time consuming process [1], which makes the final cost of this vaccine very expensive. Moreover, efforts to develop effective and safe vaccines against mycoplasmas have been partially successful [4]. As a result, vaccines developed using recombinant DNA technology become a viable alternative. Following the discovery of bacterial adhesins, the development of a bacterial adhesin vaccine through recombinant techniques has been considered [5,6]. Adherence of *M. hyopneumoniae* to the swine cilia is mediated by R1 repeat region (AAKPV-E) of the P97 adhesin [7], which is recognized by monoclonal antibody (MAb) F1B6 [8]. This MAb blocked *M. hyopneumoniae* adherence to porcine cilia in the in vitro adherence assay [9], suggesting the importance of the P97 in disease pathogenesis. P97 adhesin is an immunodominant antigen able to

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trigger early specific antibody responses in pigs following initial infection with *M. hyopneumoniae* [10,11]. Moreover, it was reported that the convalescent-phase swine sera also recognize the R1 repeat structure [7]. Cilium adhesion is a critical component of the *M. hyopneumoniae* virulence repertoire [12].

M. hyopneumoniae initiate disease following interaction with the mucosal surface lining the respiratory tract. The primary defense of this tissue is the mucosal immune system. Mucosal immune responses are characterized by the production of secretory immunoglobulin type A (sIgA). This immunoglobulin prevents the interaction of the pathogens with receptors on the mucosal cell surface. The induction of peripheral immune responses by parenteral immunization does not result in significant mucosal immunity (sIgA); however, mucosal immunization is capable of inducing protective immunity in both external secretions and peripheral immune compartments [13]. Heat-labile enterotoxin B subunit from *Vibrio cholerae* and *Escherichia coli* (CTB and LTB, respectively) have been evaluated as molecular adjuvants [14]. These non-toxic subunits exhibit more than 80% sequence identity and are potent mucosal adjuvants [14–16], stimulating a strong systemic response and a secretory response of antibodies against co-administrated or coupled antigens. LTB is a more potent mucosal adjuvant than its closely related homologue, the CTB [17,18]. LTB forms a pentamer arranged in a cylinder-like structure, with a central cavity that exposes five symmetrical cavities that are responsible for binding to the cell surface receptor, the GM1 ganglioside [19]. Adjuvanticity of LTB was found to be directly related to GM1 ganglioside-binding activity [20,21]. Recombinant LTB fused with recombinant antigens may represent an alternative in development of mucosal vaccines. Protective immunity in mice against respiratory syncytial virus [22], ocular herpes simplex virus type-1 [23], group A *Streptococcus* [24] and *Helicobacter pylori* [25], was reported using recombinant LTB or CTB as mucosal adjuvant. If high anti-R1 IgA levels could be produced at the swine respiratory surface, protection against colonization of the *M. hyopneumoniae* might be possible.

The objective of this study was to develop and to evaluate a recombinant subunit vaccine (rLTBR1) containing the R1 repeat region of P97 adhesin of *M. hyopneumoniae* (R1) fused to the B subunit of the heat-labile enterotoxin of *E. coli* (LTB). The local, systemic and cellular immunity of mice inoculated with rLTBR1 was estimated.

2. Materials and methods

2.1. Reagents

The MAb F1B6 (anti-R1) was obtained from Dr. Eileen Thacker (Iowa State University, Ames, IA, USA) and has been previously characterized [11]. *M. hyopneumoniae* 7448 pathogenic strain and swine serum positive for 7448 strain were obtained from Centro Nacional de Pesquisa de Suínos e Aves (CNPSA-EMBRAPA, Concórdia, Brazil). Mouse monoclonal antibody isotyping reagents, bovine ganglioside GM1, LPS of *E. coli*, cholera toxin, rabbit IgG anti-cholera toxin, goat IgG anti-rabbit IgG peroxidase conjugated, rabbit IgG anti-goat IgG peroxidase conjugated, MAb anti-6XHis alkaline phosphatase conjugated, goat IgG anti-mouse Igs peroxidase, rabbit IgG anti-swine Igs peroxidase conjugated and rabbit IgG anti-mouse IgA peroxidase conjugated were obtained from Sigma (USA). Reagents for sequencing were obtained from Amersham Biosciences (USA) and the BD OptEIA™ Mouse IFN-γ ELISA kit from BD Biosciences Pharmingen (USA).

2.2. Strains and DNA extraction

The genomic DNA from *M. hyopneumoniae* 7448 strain, isolated from swine with mycoplasmal pneumonia and gently provided by CNPSA, was prepared according to the method of Sambrook and Russell [26]. This strain had the entire genome sequenced by Genoma Sul project (www.genesul.lncc.br). Enterotoxigenic *E. coli* (EEC) strain, isolated from swine with diarrhea, was obtained from Centro de Biotecnologia, UFPel (Pelotas, Brazil). Genomic DNA was obtained from an EEC suspension cultured in Luria–Bertani (LB) medium during overnight incubation in shaker (37 °C, 250 rpm). After incubation, the bacterial suspension was heated at 100 °C for 5 min, centrifuged at 14,000 × *g* for 1 min and the supernatant was collected and stored at −20 °C.

2.3. PCR, ltbr1 fusion and cloning of the ltbr1 and r1 in pENTR/SD vector

The R1 repeat region of *p97* gene (*r1*) and *E. coli* heat-labile enterotoxin subunit B gene (*eltb*) were amplified by PCR using Perkin-Elmer 2400 thermocycler, with the following program: 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 68 °C for 30 s, with a final extension of 68 °C for 7 min. The reactions were performed in final volume of 25 μl containing 2.5 μl of 10× buffer, 0.5 μl of 10 mM dNTP, 150 ng of each primer, 0.5 μl (1 unit) of Platinum® *Pfx* DNA polymerase (Invitrogen), 0.5 μl of 50 mM $MgSO_4$, 18 μl of Milli-Q water and 20 ng of template DNA. Primers for *ltb* and *r1* amplification were designed based on GenBank™ sequences, found under access numbers M17873 and U50901, respectively. The following primers were used in this study: R1 forward 5′-CACCATGG**GGATCC**CTACAAAAGAAG; R1 reverse 5′-GCCAAGCTTAGTAGCAACTGGT; LTB forward 5′-CACCATGGCTCCCCAGACTATTACA; and LTB reverse 5′-CT**GGATCC**CCATACTGATTGC. The LTB reverse primer and the R1 forward primer were modified allowing a *Bam*HI restriction site insertion for the correct reading frame fusion of the 3′ end of *ltb* with the 5′ end of *r1*. This approach allowed direct fusion of the genes after restric-