Protection Efficacy of the rLTB-R1 Chimera against Experimental Swine Mycoplasmal Pneumonia

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ABSTRACT

***Background*:** *Mycoplasma hyopneumoniae* is the etiological agent of the Swine Mycoplasmal Pneumonia (SMP), one of the most economically significant diseases in the swine industry worldwide. Commonly used vaccines for SMP control consist of inactivated whole cells (bacterins). These vaccines are efficacious against *M. hyopneumoniae* challenge, but do not prevent colonization by the pathogen or completely eliminate pneumonia. P97 adhesin is conserved in the *M. pneumoniae* virulent strains, therefore it is an attractive target to be used in recombinant vaccines against *M. hyopneumoniae*. The aim of the present study was to evaluate protection afforded by rLTB-R1, a recombinant chimera composed by LTB fused with the R1 repeat region of P97 adhesin of *M. hyopneumoniae*, in specific-pathogen-free (SPF) piglets vaccinated by intranasal or intramuscular route and challenged with a pathogenic strain of *M. hyopneumoniae*.

***Materials, Methods & Results*:** PCR products of the LTB and R1 coding sequences were fused, then cloned into pETD-EST42™ expression vector. The rLTB-R1 was expressed in *Escherichia coli* BL21 (DE3) Salt induction (SI). The piglets were divided into three groups: four piglets were intranasally vaccinated with 1 mg of rLTB-R1 solubilized in 1 mL of PBS at 0 and 14 days (IN rLTB-R1 group); four piglets were intramuscularly vaccinated with 1 mg of rLTB-R1 solubilized in 1 mL of PBS at 0 and 14 days (IM rLTB-R1 group); three piglets were intranasally and intramuscularly inoculated with 1 mL of PBS (control group). Two weeks after the last immunization (28 day), piglets were intratracheally challenged with 10 mL of a suspension containing 10^9 color-changing unit (CCU) of pathogenic *M. hyopneumoniae* 7448 strain on three consecutive days. Until the challenge (28 days), intranasal and intramuscular vaccination with rLTB-R1 induced seroconversions of anti-R1 systemic antibodies of 1.6 and 4.6 ×, respectively. The IN rLTB-R1 group had no pulmonary lesion, rLTB-R1 conferred protection against experimental SMP. On the other hand, IM rLTB-R1 and control groups had on average 7.24% and 8.46% of pulmonary lesion, respectively, showing that intramuscular vaccination with rLTB-R1 did not confer protection.

***Discussion*:** The rLTB-R1, when intranasally administrated to mice, elicited production of anti-R1 IgA in trachea and bronchi as well as specific Th1 response, suggesting an adequate stimulation of the mucosal immune system. We believe that rLTB-R1 induced a similar immune response in piglets intranasally vaccinated, conferring protection against experimental SMP. The present study, the rLTB-R1 alone, without any chemical adjuvant, stimulated a significant seroconversion of anti-R1 systemic antibodies in pigs intramuscularly vaccinated, showing the potential of LTB as a parenteral adjuvant in swine vaccination. Previous work has shown that the intramuscular administration route was evaluated in pigs because mice intramuscularly vaccinated with rLTB-R1 presented significant levels of anti-R1 IgA in trachea and bronchi, suggesting that rLTB can stimulate some degree of mucosal immunity even if not delivered by a mucosal route. However, in the present study, piglets intramuscularly vaccinated with rLTB-R1 presented high levels of anti-R1 systemic antibodies, they were not protected. On the other hand, intranasal vaccination of piglets with rLTB-R1 elicited low levels of anti-R1 systemic antibodies (1.6 × at 28 days), but it conferred full protection against experimental SMP. The present study demonstrated that intranasal vaccination of piglets with rLTB-R1 conferred protection against experimental SMP. A more detailed analysis of the protective immune response induced by rLTB-R1 in pigs is currently being performed.

Keywords: *Mycoplasma hyopneumoniae*, Swine Mycoplasmal Pneumonia, mucosal vaccination, P97 adhesin, LTB.

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INTRODUCTION

Mycoplasma hyopneumoniae is the etiological agent of the Swine Mycoplasmal Pneumonia (SMP), one of the most economically significant diseases in the swine industry worldwide. Adherence of *M. hyopneumoniae* to the swine respiratory epithelial cells causes reduction of ciliary activity, ciliostasis and loss of cilia [3,22,24-26].

Adherence of *M. hyopneumoniae* to the swine cilia is mediated by R1 repeat region (AAKPV-E) of the P97 adhesin [12], which has been shown to be highly immunogenic for pigs [17,19]. P97 adhesin is conserved in the *M. pneumoniae* virulent strains, therefore it is an attractive target to be used in recombinant vaccines against *M. hyopneumoniae* [28]. However, King *et al*. [9] observed that recombinant P97 did not protect swine from becoming infected with *M. hyopneumoniae* nor reduced the severity of the disease as measured by lung lesions. It is conceivable that failure in protection was due to the route of vaccination used (intramuscular), since parenteral administration of immunogens does not induce an adequate mucosal immunity [29]. An alternative to this problem is the utilization of mucosal adjuvants. B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) has been shown to be a potent mucosal adjuvant, stimulating a strong systemic and secretory response of antibodies against co-administrated or coupled antigens [2,7,13,15].

The aim of the present study was to evaluate protection afforded by rLTB-R1, a recombinant chimera composed by LTB fused with the R1 repeat region of P97 adhesin of *M. hyopneumoniae*, in specific-pathogen-free (SPF) piglets vaccinated by intranasal or intramuscular route and challenged with a pathogenic strain of *M. hyopneumoniae*.

MATERIALS AND METHODS

Cloning, expression, and purification of recombinant ETX

The rLTB-R1 was constructed, expressed and characterized as previously described [7]. Briefly, PCR products of the LTB and R1 coding sequences were fused, then cloned into pENTR/SD cloning plasmid[1]. The fragment was then transferred by site specific recombination to pETDEST42™ expression vector[1], which allows fusion of the protein with a C terminal 6 × His tag. After transformation of the construct into an appropriate *E. coli* BL21 (DE3) SI, and induction with NaCl and isopropyl β-D-1-thiogalactopyranoside (IPTG), the recombinant protein was purified by Ni-NTA affinity chromatography, quantified by the Bradford method and characterized by Western blot.

Bacterial strain

The pathogenic *Mycoplasma hyopneumoniae* 7448 homologous strain was used in the challenge experiment. This strain was grown in Friis medium[2]. Cultures consisted of 125 mL of Friis medium in Erlenmeyer flasks incubated at 37°C with slow agitation until the culture reached mid-log phase, as indicated by color change and turbidity.

Experimental design

Eleven 6 to 8-week-old SPF piglets were obtained from Embrapa Suínos e Aves, Concórdia, SC, Brazil. These animals had no serological evidence of exposure to *M. hyopneumoniae* as examined by an ELISA using Tween 20 soluble proteins of *M. hyopneumoniae* as antigens [16,18]. They were allocated into three groups: four piglets were intranasally vaccinated with 1 mg of rLTB-R1 solubilized in 1 mL of PBS at 0 and 14 days (IN rLTB-R1 group); four piglets were intramuscularly vaccinated with 1 mg of rLTB-R1 solubilized in 1 mL of PBS at 0 and 14 days (IM rLTB-R1 group) and; three piglets were intranasally and intramuscularly inoculated with 1 mL of PBS (control group). Two weeks after the last immunization (28 day), piglets were intratracheally challenged with 10 mL of a suspension containing 10^9 color-changing unit (CCU) of pathogenic *M. hyopneumoniae* 7448 strain on three consecutive days. Food and water were provided *ad libitum*. Eight weeks after the challenge exposure, the piglets were euthanatized and necropsied. For determination of the extent of gross lung lesions, the percentage of lung affected by pneumonia was calculated following Morrison *et al*. [14]. Lung samples were microscopically examined for characteristic mycoplasmal pneumonia histopathological damage as previously described [25]. Slides with lung tissue (3-5 μm thick) were also used for immunohistochemistry using polyclonal sera against *M. hyopneumoniae*, produced by Embrapa Suínos e Aves, and Vectastain ABC kit[®3]. Briefly, paraffin was removed from the slides and tissue rehydrated. Antigen was recovered with citrate buffer (pH 6,0) and heat (microwave oven for 5 min, 700 W) and enzymatically digested with pepsin (0.04% in HCl 0.001 N - pH 7.8) for 10 min at 37°C.

Endogenous peroxidase was inactivated with hydrogen peroxide (3%) for 5 min. Slides were incubated with the primary antibody for 2 h at 37°C, followed by binding to the Vectastain ABC kit[®3]. Reaction was developed with 3-amino-9-ethylcarbazole (AEC) for 5 min at 37°C. Mayer's hematoxylin was used to conter stain the slides. Slides were washed for 5 min between each step with PBS (pH 7.4) plus Tween 20 (0.05%). Slides were read with an optic microscope.

Sample sera were weekly collected. Seroconversions of anti-R1 systemic antibodies determined by ELISA and Western blot analysis were performed as previously described [7]. Mean seroconversion of anti-R1 systemic antibodies, pulmonary lesion scores and immunohistochemistry scores were subjected to analysis of variance. The following criteria were used in statistical analysis of pulmonary lesions and immunohistochemistry, respectively: score 1 for presence of pulmonary lesion and 0 for absence; imunohistocemestry score 0: absence of staining; weak (1 cross), moderate (2 crosses), and strong (3 crosses) staining in ciliated epithelium of bronchia and bronchioles [20]. The Student's *t*-test was used to determine significant differences ($P < 0.05$) among the means of each group through the Statistix software.

RESULTS

The IN rLTB-R1 group had no pulmonary lesion, therefore intranasal vaccination with rLTB-R1 conferred full protection against experimental SMP ($P < 0.001$ in comparison with IM rLTB-R1 and control groups). On the other hand, IM rLTB-R1 and control groups had on average 7.24% and 8.46% of pulmonary lesion, respectively, showing that intramuscular vaccination with rLTB-R1 did not confer protection. Immunohistochemistry assay showed that none of the piglets of the IN rLTB-R1 group had evidence of infection ($P < 0.001$ in comparison with IM rLTB-R1 and control groups), while all animals of the IM rLTB-R1 and control groups were positive (Figure 1). Table 1 shows the results of the challenge test.
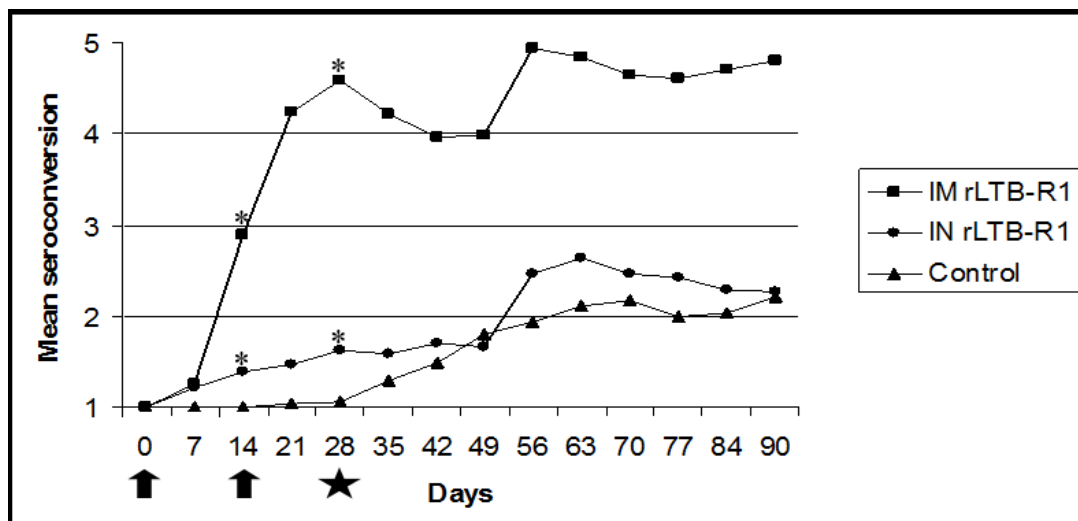
Mean seroconversion of anti-R1 systemic antibodies is shown in Figure 2. Both intranasal and intramuscular vaccination with rLTB-R1 induced significant seroconversions ($P < 0.05$) at 14 and 28 days in comparison with the control group, showing that the recombinant chimera was immunogenic to pigs. Until the challenge (28 days), intranasal and intramuscular vaccination with rLTB-R1 induced seroconversions of anti-R1 systemic antibodies of 1.6 and 4.6 ×, respectively. Four weeks after the challenge (56 days), seroconversions of anti-R1 systemic antibodies of all groups increased, suggesting immunologic stimulation by the challenge strain. Western blot analysis showed that the pooled sera from piglets intramuscularly vaccinated with rLTB-R1 recognized the P97 adhesin of the *M. hyopneumoniae* 7448 pathogenic strain and the products derived from its proteolytic processing (data not shown).

Table 1. Lesion score and amount of antigen by immunohistochemistry (IHC) in bronchioles of different treatment groups after challenge with pathogenic *Mycoplasma hypneumoniae* strain 7448.

Groups	% Lesions	Immunohistochemistry
IM rLTB-R1		
Piglet 1	3.12	+++
Piglet 2	23.87	+++
Piglet 3	1.0	++
Piglet 4	1.0	+
Mean	7.24	
IN rLTB-R1		
Piglet 1	0	-
Piglet 2	0	-
Piglet 3	0	-
Piglet 4	0	-
Mean	0	
Control		
Piglet 1	1.0	+
Piglet 2	23.37	+++
Piglet 3	1.0	+
Mean	8.46	
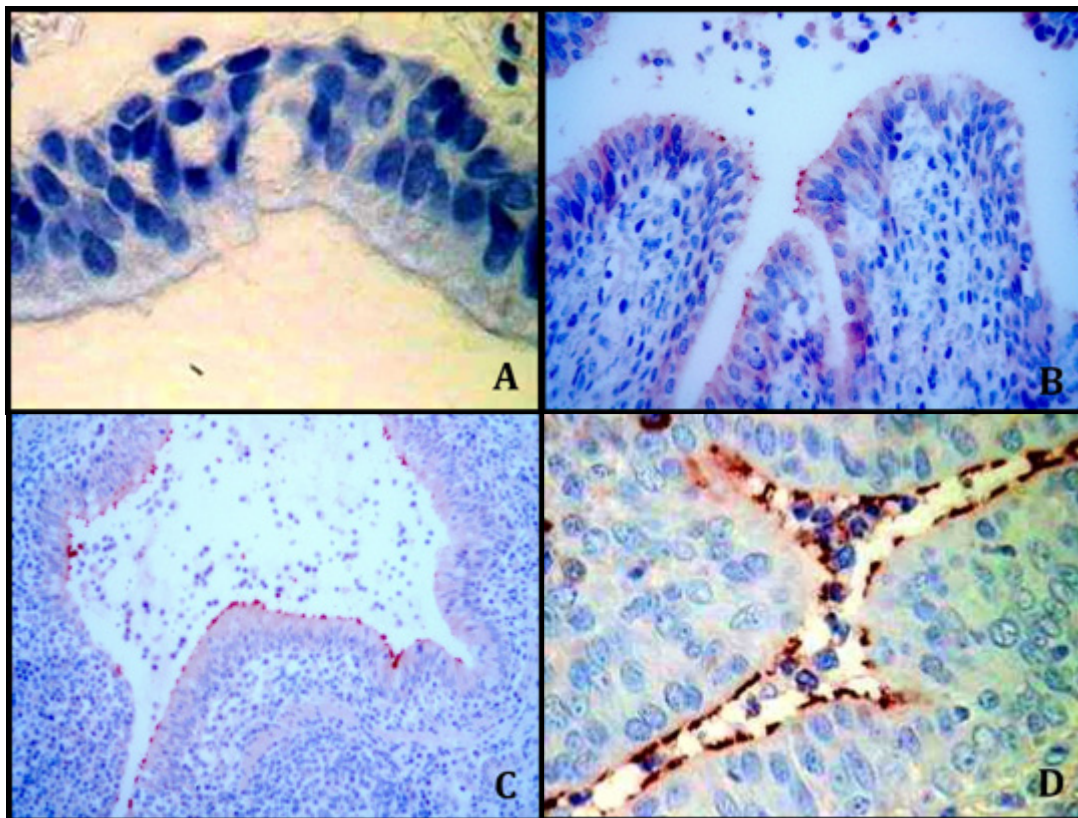
Figure 1. Immunohistochemistry scores in bronchioles of animals challenged with *Mycoplasma hyopneumoniae* strain 7448. A- Absence of staining in cilia of epithelial cells [100x]. B- Positive staining (+) in cilia of epithelial cells [50x]. C- Positive staining (++) in cilia of epithelial cells [40x]. D- Positive staining (+++) in cilia of epithelial cells [100x]. Positive staining is represented by red colour.

Figure 2. Mean seroconversion of anti-R1 systemic antibodies determined by ELISA using rR1 as antigen. Piglets were intranasally or intramuscularly vaccinated with 1 mg of rLTB-R1 at 0 and 14 days (arrows). Two weeks after the last immunization, piglets were intratracheally challenged with 10^9 CCU of pathogenic *Mycoplasma hyopneumoniae* 7448 strain. *$P < 0.05$ in comparison to the control group.

DISCUSSION

In the present study we evaluated protection induced by the rLTB-R1 chimera, a new vaccine candidate for controlling SMP composed by the B subunit of *E. coli* heat-labile enterotoxin fused with the R1 repeat region of the P97 adhesin of *M. hyopneumoniae*. Commonly used vaccines to SMP control consist of inactivated whole cell preparations. These vaccines

can provide protection for pigs against experimental *M. hyopneumoniae* challenge but cannot completely eliminate pneumonia or significantly reduce colonization by the microorganism. Inactivated whole cell vaccines are efficacious in the prevention of infectious diseases controlled by systemic antibodies; however, are incapable of inducing a suitable mucosal immunity [5,15].

The rLTB-R1, when intranasally administered to mice, elicited production of anti-R1 IgA in trachea and bronchi as well as specific Th1 response [7], suggesting an adequate stimulation of the mucosal immune system. We believe that rLTB-R1 induced a similar immune response in piglets intranasally vaccinated, conferring protection against experimental SMP. Several researchers suggested that mucosal (IgA) and cellular immune responses may be important in the resolution of *M. hyopneumoniae* infection [6,7,11,18]. Although the evaluation of the humoral and/or cellular immune responses induced by an experimental vaccine is of interest, the most important parameter to be evaluated is the level of protection afforded by the vaccine candidate.

To stimulate a significant systemic IgG response against the R1 region of the P97 adhesin of *M. hyopneumoniae* in pigs, the PE(ΔIII)-RR1 recombinant chimera, composed by *Pseudomonas* exotoxin fused with the R1 repeat region of the P97 adhesin of *M. hyopneumoniae*, needed to be adjuvanted with aluminum hydroxide and co-administrated with a commercial bacterin against SMP [4]. Galli *et al*. [8] evaluated five *M. hyopneumoniae* antigens (P37, P42, P46 e P95) as vaccine in mice, of which, P42, P95 and pcDNA3/P46 were effective of generating immune response. DNA vaccine pcDNA3/P46 induced antibodies IgG1 e IgG2a, as well as higher levels of INFγ compared with subunit vaccines. Studies by Chen *et al*. [6] using DNA vaccines containing antigens P36, P46, NrdF, P97 or P97R1, antigen P46 generated mainly IgG. The present study, the rLTB-R1 alone, without any chemical adjuvant, stimulated a significant seroconversion of anti-R1 systemic antibodies in pigs intramuscularly vaccinated, showing the potential of LTB as a parenteral adjuvant in swine vaccination.

The intramuscular administration route was evaluated in pigs because mice intramuscularly vaccinated with rLTB-R1 presented significant levels of anti-R1 IgA in trachea and bronchi [7], suggesting

that rLTB can stimulate some degree of mucosal immunity even if not delivered by a mucosal route. Similar results were obtained [27] using rLTB as adjuvant and urease of *Helicobacter pylori* as immunogen. Although piglets intramuscularly vaccinated with rLTB-R1 presented high levels of anti-R1 systemic antibodies, they were not protected. Similarly, intramuscular vaccination of piglets with recombinant GST-P97 using complete Freund's adjuvant elicited a strong systemic humoral response, but it was incapable of protecting against experimental SMP [9]. On the other hand, intranasal vaccination of piglets with rLTB-R1 elicited low levels of anti-R1 systemic antibodies (1.6 × at 28 days), but it conferred full protection against experimental SMP. Other reports showed that serum antibodies induced by commercial *M. hyopneumoniae* vaccines do not correlate with protection against *M. hyopneumoniae* challenge [10,23]. Therefore, serum antibodies may not play a protective role in SMP, suggesting that an adequate stimulation of the mucosal immune system is important in SMP prevention. In human and chickens, a positive correlation between anti-*Mycoplasma* specific IgA levels and protection has been established [1,21].

Recombinant vaccines using P97 adhesin as antigen have been evaluated. Ogawa *et al*. [17] demonstrated *Erysipelothrix rhusiopathiae* 65-0.15 strain expressing on the surface the C-terminal portion of *M. hyopneumoniae* P97 adhesin, orally administrated to piglets, reduced the severity of pneumonic lung lesions caused by experimental SMP. It was demonstrated that mice immunized orally with attenuated *Salmonella* typhimurium aroA strain CS332 harboring a eukaryotic or prokaryotic expression vector encoding P97 adhesin did not produce a local and systemic humoral response against P97 [5]. Okamba *et al*. [18] showed a significant anti-P97 immune response in mice intranasally vaccinated with a replication-defective recombinant adenovirus expressing the C-terminal portion of P97 adhesin. The vaccine candidates against SMP previously mentioned use attenuated pathogens as vectors, a strategy whose tendency is to disappear.

The present study demonstrated that intranasal vaccination of piglets with rLTB-R1 conferred protection against experimental SMP. A more detailed analysis of the protective immune response induced by rLTB-R1 in pigs is currently being performed.

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