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A novel chimeric protein composed of recombinant *Mycoplasma hyopneumoniae* antigens as a vaccine candidate evaluated in mice

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ABSTRACT

Enzootic Pneumonia (EP) is caused by the *Mycoplasma hyopneumoniae* pathogenic bacteria, and it represents a significant respiratory disease that is responsible for major economic losses within the pig industry throughout the world. The bacterins that are currently commercially available have been proven to offer only partial protection against *M. hyopneumoniae*, and the development of more efficient vaccines is required. Several recombinant antigens have been evaluated via different immunization strategies and have been found to be highly immunogenic. This work describes the construction and immunological characterization of a multi-antigen chimera composed of four *M. hyopneumoniae* antigens: P97R1, P46, P95, and P42. Immunogenic regions of each antigen were selected and combined to encode a single polypeptide. The gene was cloned and expressed in *Escherichia coli*, and the chimeric protein was recognized by specific antibodies against each subunit, as well as by convalescent pig sera. The immunogenic properties of the chimera were then evaluated in a mice model through two recombinant vaccines that were formulated as follows: (1) purified chimeric protein plus adjuvant or (2) recombinant *Escherichia coli* bacterin. The immune response induced in BALB/c mice immunized with each formulation was characterized in terms of total IgG levels, IgG1, and IgG2a isotypes against each antigen present in the chimera. The results of the study indicated that novel chimeric protein is a potential candidate for the future development of a more effective vaccine against EP.

1. Introduction

Enzootic pneumonia (EP), which results from *M. hyopneumoniae* infection, is a respiratory disease that is highly prevalent within the intensive pig farming industry. The bacteria is typically inhaled into the respiratory tract where it begins the infection process by binding to the cilia of epithelial cells in the airways, eliciting the dysfunction of cilia and leading to ciliary clearance (DeBey and Ross, 1994; Thacker et al., 2000). The clinical progression of EP is mainly characterized by chronic, non-productive cough, reduced rate of average daily weight gain, and reduced feed conversion efficiency (Maes et al., 2008; Sibila et al., 2009). The decrease in productivity and the cost of medication used to treat this disease causes significant economic losses to the swine industry throughout the world (Haesebrouck et al., 2004; Maes et al., 2008; Sibila et al., 2009).

Vaccination represents the most used strategy through which EP can be controlled (Haesebrouck et al., 2004). The current commercial vaccines consist of inactivated whole-cell adjuvanted formulations and are used throughout the world (Maes et al., 2008; Sibila et al., 2009). Although they improve productive performance, these vaccines do not prevent the pathogen from colonizing the respiratory tract and do not induce sterilizing immunity; as such, they provide only partial protection against the disease (Haesebrouck et al., 2004; Maes et al., 2008; Thacker et al., 2000). In order to identify an alternative vaccine that can overcome the limitations associated with the bacterins that are currently available, a reverse vaccinology approach has been used. Data generated by sequencing five strains of *M. hyopneumoniae* (232, J, 7448, 7422, and 168) (Liu et al., 2013; Minion et al., 2004; Siqueira et al., 2013; Vasconcelos et al., 2005) allowed the identification and

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characterization of several recombinant antigens with immunogenic potential for use in more effective vaccines.

A few recombinant antigens from *M. hyopneumoniae* have been extensively studied by several groups. The C-terminal region of adhesin P97 (P97R1), which has been found to be among the most promising antigens, exhibits an important virulence factor that is involved in the pathogen's adherence to the host respiratory tract (Conceição et al., 2006; Marchioro et al., 2014a). The molecular chaperone DnaK (P42), a heat shock protein that is a member of the HSP70 family, is highly expressed in stress conditions (Galli et al., 2012; Jorge et al., 2014; Marchioro et al., 2014a; Simionatto et al., 2012). The outer membrane protein P95 and the 46-kDa membrane surface protein (P46), which are possibly exposed on the pathogen surface, are strongly recognized by convalescent pig sera (Galli et al., 2012; Simionatto et al., 2012). Different approaches by which the immunogenicity of recombinant vaccines can be enhanced have been evaluated. Constructions based on multi-antigens represent an interesting strategy for combining proteins from one or more pathogens in a single molecule (Chen et al., 2008; Marchioro et al., 2014b).

In this study, we developed a chimeric protein composed of four *M. hyopneumoniae* antigens: P97R1, P46, P95, and P42. The antigenicity of this construction was verified using serum from naturally infected pigs. In addition, the immunogenicity of this chimera was evaluated by testing different vaccine formulations in mice.

2. Materials and methods

2.1. In silico *selection of coding sequences and gene design*

Coding DNA sequences (CDS) and protein sequences for P97R1 (MHP_0198), P46 (MHP_0513), P95 (MHP_0099), and P42 (MHP_0067) antigens from *M. hyopneumoniae* strain 7448 (NC007332) were used as reference to design the chimeric gene (Table 1). These sequences were analyzed using the following bioinformatics software: SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/), TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/), IEDB-Antibody Epitope Prediction (http://tools.iedb.org/bcell/) and Vector NTI Advance® 11 (Invitrogen™). Regions encoding surface-exposed, predominantly hydrophilic and with a high number of linear epitopes were selected. Restriction sites for *Bam*HI and *Kpnl* were added flanking the gene, and a flexible linker Gly_{2x}SerGly was inserted between each gene portion to enable the proper folding of protein. The *in silico* protein structure was constructed using the I-TASSER online server (Zhang, 2008) and visualized using UCSF Chimera package software (Pettersen et al., 2004).

2.2. Cloning, expression, and purification of recombinant proteins

The *p97r1p46p95p42* gene was chemically synthesized (Epoch Biolabs, Inc., USA) and provided on pBluescript II SK(−) (pBSK) vector. The gene was excised from pBSK plasmid with restriction enzymes and cloned into the pAE vector (Ramos et al., 2004) using *E. coli* Top 10 (Invitrogen) competent cells as previously described (Sambrook and Russell, 2001). The pAE/*p97r1p46p95p42*, pAE/*p97r1*, pET/*p46*, pAE/*p95*, and pAE/*p42* recombinant plasmids were transformed in *E. coli* BL21 (DE3) Star (Invitrogen) cells. The expression and purification of recombinant proteins were performed using a previously described method (Conceição et al., 2006; Marchioro et al., 2012).

2.3. Antigenicity assays of chimeric protein

To assess the antigenicity of the chimeric protein, Western blot and ELISA were performed using the previously described method (Simionatto et al., 2012; Jorge et al., 2014). For Western blot, mouse antibodies and dilutions were used as follows: monoclonal anti-6xHis (Sigma-Aldrich), diluted 1:6000; monoclonal anti-P97R1 (F1B6), diluted 1:4000 (Dr. Eileen Thacker– Iowa State University, USA); polyclonal anti-P46, anti-P95, and anti-P42, diluted 1:50 (previously produced) (Galli et al., 2012); and HRP-conjugated goat anti-mouse (Sigma-Aldrich), diluted 1:6000. For indirect ELISA, microtiter plates were coated using 50 ng/well of purified chimeric protein. Sera samples from specific pathogen-free (SPF) (n = 32) and convalescent phase (n = 105) pigs, diluted at a ratio of 1:50, were obtained from a mycoplasma-negative farm and from three commercial herds that had been chronically affected by EP in Southern Brazil, respectively.

2.4. Vaccine formulations and mice vaccination

The purified chimeric protein was diluted in oil-adjuvant AddaVax™ (InvivoGen) at a ratio of 1:1 according to the manufacturer's instructions. Recombinant bacterins were produced in *E. coli* BL21 (DE3) Star as per the method previously described (Moreira et al., 2016). Female BALB/c mice that were aged eight weeks were allocated to five different experimental groups (Table 2) with six animals in each group, as follows: (1) rP97R1P46P95P42 + AddaVax™ adjuvant (rCHI Add); (2) Negative control A, PBS + AddaVax™ adjuvant (PBS Add); (3) *E. coli* bacterin expressing rP97R1P46P95P42 (*E. coli* rCHI); (4) Negative control B, *E. coli* bacterin containing pAE vector (*E. coli* Vec); and (5) Positive control, Sprintvac MH (Merial) (BACT). Two doses of 50 μg of purified protein or *E. coli* bacterins were administered intramuscularly (IM) at a 21-day interval. Blood samples were collected from retro-orbital sinus 0, 21, 42, and 63 days after the first inoculation (DAI), and stored at −20 °C before being processed. All animal experiments were conducted in accordance with the recommendations of the Ethics Committee for Animal Experimentation of the Federal University of Pelotas (Permit number: 2351-2015).

2.5. Total IgG antibodies and isotype profile against recombinant proteins

Specific antibodies induced by mice immunization with vaccine formulations were assessed by ELISA and Western blot using the process previously described (Simionatto et al., 2012), with some modifications. For ELISA, microtiter plates were coated with chimeric protein (100 ng/well) or each chimeric subunit protein (rP97R1, rP95 and rP42: 100 ng/well; rP46: 50 ng/well). To determine total IgG, proteins were incubated with pooled serum

Table 1
Characteristics of antigens select in the chimeric protein.

Protein name	Annotation genome strain 7448	NCBI accession number	Features/function	Original size (kDa)	Selected fragment (aa)
P97R1	MHP_0198	YP_287595.1	Adhesin P97	122	788–915
P46	MHP_0513	YP_287902.1	46 K surface antigen precursor	46	323–419
P95	MHP_0099	YP_287499.1	Outer membrane protein – P95	132	604–750
P42	MHP_0067	YP_287467.1	Molecular chaperone DnaK	65	434–600