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The *Mycoplasma hyopneumoniae* recombinant heat shock protein P42 induces an immune response in pigs under field conditions

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ABSTRACT

Enzootic pneumonia (EP), resulting from *Mycoplasma hyopneumoniae* infection is one of the most prevalent diseases in pigs and is a major cause of economic losses to the swine industry worldwide. EP is often controlled by vaccination with inactivated, adjuvanted whole-cell bacterin. However, these bacterins provide only partial protection and do not prevent *M. hyopneumoniae* colonization. Attempts to develop vaccines that are more efficient have made use of the recombinant DNA technology. The objective of this study was to assess the potential of recombinant *M. hyopneumoniae* heat shock protein P42 in vaccine preparations against EP, using piglets housed under field conditions in a *M. hyopneumoniae*-positive farm. The cellular and humoral immune responses were elicited after a single intramuscular inoculation of rP42 in an oil-based adjuvant, or in conjunction with whole-cell vaccine preparation. The production of INF-γ and IL-10 cytokines was quantified in the supernatant of the cultured mononuclear cells. The rP42 emulsified in oil-based adjuvant was able to trigger a strong humoral immune response. Further, it induced a cellular immune response, accompanied by the production of antibodies that reacted with the native *M. hyopneumoniae* protein. The rP42 mediated induction of cellular and humoral immune response in the host suggests that rP42 emulsified in an oil-based adjuvant holds promise as an effective recombinant subunit vaccine against EP.

1. Introduction

Mycoplasma hyopneumoniae is the causative agent of enzootic pneumonia (EP), a highly prevalent respiratory disease, responsible for significant economic losses to the swine industry. This disease is characterized by chronic non-productive coughing, poor growth rate, low feed conversion, and delayed weight gain, with high morbidity and low mortality. In addition, EP increases the host's susceptibility to other respiratory pathogens and secondary infections [1,2].

Vaccination with inactivated, adjuvanted whole-cells is frequently used worldwide to control *M. hyopneumoniae* infections [3], but their efficiency is questionable. The

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production costs of these vaccines are very high because of the difficulties associated with the *in vitro* cultivation of *M. hyopneumoniae* [4]. Furthermore, they provide only partial protection [4,5] and are unable to prevent the transmission or establishment of the microorganism in the lungs [6,7]. Thus, there is a need to develop more effective and less expensive vaccines against EP. Recombinant DNA technology can be useful in overcoming the common problems encountered with conventional vaccines, and the small genome of this pathogen, as well as the limited number of secreted or surface proteins, favors the use of reverse vaccinology approach [8].

The genomes of four strains of *M. hyopneumoniae* have been sequenced [9–11] and proteomic analysis has been performed for two *M. hyopneumoniae* strains [11]. Our research group produced 35 secreted [12,13] and six transmembrane recombinant proteins [14] expressed in *Escherichia coli*, and evaluated their immunogenic and antigenic properties. A few of these proteins were identified as potential vaccine antigens, including well-known immunodominant antigens found in several bacterial species, such as the heat shock protein (Hsp) family members that are highly conserved in prokaryotes [15,16]. One such Hsp family member is the *M. hyopneumoniae* molecular chaperone DnaK (P42 heat shock protein) that has been shown to induce strong immune responses in a mice model [12,17] and was recognized by sera from convalescent pigs [12].

In this work, the cellular and humoral immune responses stimulated by recombinant heat shock protein P42 (rP42) subunit vaccine preparations were evaluated in piglets under field conditions in a *M. hyopneumoniae*-positive farm.

2. Materials and methods

2.1. Cloning, expression, and purification of rP42

Genomic DNA extraction of *M. hyopneumoniae* strain 7448 was performed using the illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The *p42* gene (MHP0067) was amplified using the primer pair P42_F (5′-taGGATCCATGGCGCTTACAAGAC) and P42_R (5′-cggGGTACCTTAATCCTGCTTG). The PCR mixture consisted of 50 ng of *M. hyopneumoniae* genomic DNA, 0.2 mM dNTP, 2.5 mM $MgCl_2$, 10 pmol of each primer, 2.5 units of Platinum *Pfx* DNA polymerase (Life Technologies, New York, USA), 1× reaction buffer and 1× enhancer buffer in a final volume of 25 μL. DNA amplification was performed using a Gradient Mastercycler (Eppendorf, New York, USA) with the following settings: 7 min at 95 °C, followed by 30 cycles of 60 s at 95 °C, 60 s at 55 °C, and 60 s at 68 °C, and then a final extension of 7 min at 68 °C. The amplicon was cloned into pAE expression vector [18], and the ligation products were transformed *E. coli* TOP10 electro competent cells. Recombinant clones were identified by agarose gel electrophoresis of extracted plasmid DNA and confirmed by restriction enzyme digestion. The integrity of the insert was confirmed by DNA sequencing using the DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis Systems – MegaBACE 500 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The rP42 protein was expressed in *E. coli* BL21 (DE3) Star and purified by affinity chromatography using HisTrap™ HP 1 mL columns prepacked with precharged Ni Sepharose™ (GE Healthcare) as previously described [12]. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Scientific, Rockford, IL, USA).

Table 1
Groups of piglets and vaccine preparations used in the experiment.

Group	Immunogen	Dose	Route
Group 1	Oil-adjuvant[a] (control)	2 mL	i.m.
Group 2	Respisure-One®	2 mL	i.m.
Group 3	Respisure-One® + rP42	2 mL + 100 μg	i.m.
Group 4	Oil-adjuvant[a] + rP42	2 mL + 100 μg	i.m.
Group 5	PBS + rP42	2 mL + 100 μg	i.m.

[a] Mineral oil based adjuvant Montanide™ ISA 50 V2; i.m., intramuscular injection.

2.2. Western blot analysis

The purified rP42 was separated on 12% SDS-PAGE, electrotransferred onto a nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and blocked with 5% non-fat dry milk in phosphate buffered saline (PBS) at 37 °C for 2 h. Two Western blot analyses, one using an anti-6′ His antibody (Sigma–Aldrich, Saint Louis, Missouri, USA), and another using a polyclonal anti-rP42 antibody, previously produced in mice [12] were performed to confirm the presence of the purified protein, to determine the apparent molecular mass of the recombinant protein, and to assess the antigenicity. After washing with PBS containing 0.05% (v/v) Tween® 20 (PBS-T), the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG monoclonal antibody. Immunoreactive protein bands were detected with 0.005% (w/v) 4-chloro-1-naphthol and 0.015% (v/v) hydrogen peroxidase in PBS.

2.3. Experimental design

The study was performed after obtaining the approval of the Ethical Committee for Animal Experiments of the Universidade Federal de Pelotas (approval number 5614). All the animals belonged to a commercial farm in Southern Brazil and were maintained under field conditions. The herd was screened using a commercial indirect ELISA kit (IDEXX *M. hyo*. antibody ELISA, Westbrook, Maine, USA) and nested PCR. Forty piglets at 21 days of age were randomly allocated into five treatment groups (8 animals per group) and immunized intramuscularly (IM) with a single dose of the following: group 1, oil-adjuvant only (Montanide ISA 50 V2®, SEPPIC, Paris, France); group 2, commercial *M. hyopneumoniae* inactivated whole-cell vaccine (RespiSureOne®, Pfizer Animal Health, Florham Park, New Jersey, USA); group 3, RespiSureOne® + rP42; group 4, oil adjuvant + rP42 (used at a ratio of 1:1); and group 5, PBS + rP42 (Table 1). Blood samples were collected from the jugular vein at 1, 21, 42, 63, and 84 days post inoculation (d.p.i.). The herd used was not vaccinated for EP