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Original Article

# Commercial bacterins did not induce detectable levels of antibodies in mice against *Mycoplasma hyopneumoniae* antigens strongly recognized by swine immune system

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ABSTRACT

Enzootic Pneumonia (EP) caused by *Mycoplasma hyopneumoniae* results in major economic losses to the swine industry. Hence, the identification of factors that provide protection against EP could help to develop effective vaccines. One such factor that provides partial protection are bacterins. Therefore, the aim of this study was to verify the induction of antibodies against fifteen *M. hyopneumoniae* antigens, strongly recognized by the swine immune system during natural infection, in mice vaccinated with six commercial bacterins. Each group of mice was inoculated with one bacterin, and seroconversion was assessed by indirect ELISA using recombinant antigens and *M. hyopneumoniae* 7448 whole cell extract. Sera from one inoculated group recognized antigen MHP_0067, and sera from four inoculated groups recognized antigens MHP_0513 and MHP_0580. None of the bacterins was able to induce seroconversion against the twelve remaining antigens. This absence of a serological response could be attributed to the lack of antigen expression in *M. hyopneumoniae* strains used in bacterin production. Additionally the partial protection provided by these vaccines could be due to low expression or misfolding of antigens during vaccine preparation. Therefore, the supplementation of bacterins with these recombinant antigens could be a potential alternative in the development of more effective vaccines.



## 1. Introduction

Enzootic Pneumonia (EP) is a worldwide disease that results in major economic losses to the swine industry [1]. Commercial vaccines, which consists of inactivated whole-cell adjuvanted formulations (bacterins), induce partial protection against EP [2,3]. These vaccines provide protection by reducing lung damage, clinical signs and weight loss and improving production rates in vaccinated animals [4–7]. Although the partial protective mechanisms have not been fully elucidated, it has been described that these bacterins are able to induce both local and systemic immune responses [8–10]. However, they are not capable of avoiding transmission of the pathogen [11,12]. Therefore, identification of the factors that mediate the partial protection of bacterins can enable the development of highly effective vaccines against EP.

Comparative analysis of *Mycoplasma hyopneumoniae* strains allowed the identification of virulence factors possibly related to bacterial pathogenesis [13–16]. Previous work has shown that commercial bacterins did not induce antibodies against P97 adhesin and NrdF protein [17–20], virulence factors well characterized that were capable of promoting some level of protection in swine subjected to experimental challenge conditions [21–24].

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Furthermore, in corroboration with published literature, recent studies from our group also demonstrated that one commercial bacterin did not induce antibodies in mice against several *M. hyopneumoniae* surface antigens [19,20,25,26]. Hence in order to better understand the bacterins and their protective mechanisms, it is essential to investigate the profile of antibodies generated by these formulations. Therefore, the aim of this study was to verify the induction of antibodies against fifteen *M. hyopneumoniae* antigens in mice vaccinated with six commercial bacterins. These antigens were selected based on our previous studies that demonstrated their ability to be strongly recognized by swine immune system during *M. hyopneumoniae* natural infection [20,25–27].

## 2. Materials and methods

### 2.1. Commercial vaccines

Six commercial bacterins against EP were used in this study: Ingelvac Mycoflex® (Boehringer Ingelheim; 008/10), M+Pac® (Intervet; 00051097 023/11), Mypravac® Suis (Hypra, 15FH-1 002/11), Respisure® 1One (Pfizer, 002/2011), Resprotek™ One Shot (Bayer, 002/2012) and Serkel Pneumo (Vencofarma, 002/2010). These bacterins were randomly designed as A–F.

### 2.2. Selection and production of recombinant antigens

Fifteen proteins with probability of being virulence factors were selected from the *M. hyopneumoniae* 7448 database (GenBank, NC_007332.1) [14]. The following criteria were used for candidate protein selection: (i) likelihood of being a virulence factor based on genomic and proteomic comparative analysis between virulent and avirulent *M. hyopneumoniae* strains [14–16]; (ii) predicted cellular localization on the outer cell surface (transmembrane or secreted protein) [25,27]; (iii) high antigenicity [25,26]; (iv) high immunogenicity in mice [25,26]; (v) significant probability of having adhesin activity relative to the protein MHP_0198 (P97 adhesin) according to the analisis from Vaxign software [28]. The recombinant proteins corresponding to the fifteen selected antigens were produced according to Simionatto et al. [27].

### 2.3. Swine sera

Three pools of positive and negative swine sera (three sera/pool) were used to confirm antigenicity of recombinant antigens and as ELISA controls. Positive swine sera were obtained from animals aged approximately 3 months old, unvaccinated against EP, and positive diagnosis of EP. This diagnosis was based on clinical signs, positive serology assessed by indirect ELISA with crude extract of *M. hyopneumoniae* strain 7448 [27] and positive PCR from nasal swab [29]. Negative sera were obtained from a swine herd tested to be free from *M. hyopneumoniae* infection through PCR and serology.

### 2.4. Mice inoculation

Male BALB/c mice aged 6–8 weeks old (5 mouse/group) were immunized with commercial vaccines (one bacterin/group) or saline without adjuvant (100 μl) as negative control, via intramuscular injection. The mice received 5% of the dose recommended by the manufacturer for swine. When indicated, each animal was boosted with the same dose 21 days after the first innoculation. Blood samples were collected from the retro-orbital sinus at days 0 and 42 after first innoculation. At day 42, the animals were euthanized. All animal experiments were performed according to the guidelines of the Ethics Committee in Animal Experimentation from the Federal University of Pelotas (project number 7722). The experiment was repeated twice.

### 2.5. Assessment of humoral immune response

Seroconversion of mice was evaluated through indirect ELISA using recombinant antigens or *M. hyopneumoniae* 7448 whole cell extract. A previous analysis was performed to determine the optimum amount of antigens (100 ng, 300 ng, 500 ng, 750 ng or 1 μg) to coat the plate and the mice sera diluition (1:25, 1:50, 1:100 or 1:200) to be used in the analysis. The antigen amount which resulted in highest reactivity before reaching the plateau, and the lowest sera diluition with no detection of background (detectable reactivity against negative sera) were selected to perform the ELISA. Microtiter plates were coated with recombinant antigens or with crude extract of *M. hyopneumoniae* strain 7448 (500 ng/well) diluted in 50 mM carbonate–bicarbonate (pH 9.6) and incubated overnight at 4 °C. Wells were washed three times with phosphate-buffered saline + 0.05% Tween 20 (PBS-T) and incubated for 2 h at 37 °C with 5% non-fat dry milk diluted in PBS (blocking solution). After three washes, wells were incubated for 2 h at 37 °C with mice or swine sera (1:50) diluted in blocking solution. After three washes, wells were incubated for 1 h and 30 min at 37 °C with goat IgG anti-mouse antibody (1:6000) or rabbit IgG anti-pig (1:4000) conjugated to horseradish peroxidase (Sigma Aldrich) diluted in PBS. Reaction was developed with o-phenylenediamine dihydrochloride (Sigma Aldrich) and hydrogen peroxide, after PBS-T washes. The color reaction was allowed to develop for 15 min and stopped with 50 μL of 2 M $H_2SO_4$. Absorbance was determined at 492 nm with plate reader ThermoPlate ELX800 (BioTek Instruments). The mean and standard deviation (S.D.) values for animal samples were calculated. Reactions were preformed in triplicate. Values of seroconversion were obtained by the quotient between the average absorbance of immune sera and non-immune sera (O.D. day 42/O.D. day 0 for mice and O.D. positive sera/O.D. negative sera for swine). Seroconversion was considered positive when increased four times.

### 2.6. Statistical analysis

GraphPad Prism 4 software systems (GraphPad Software) was used to perform the statistical analysis and build the graphics. Seroconversion data were submitted to ANOVA followed by Dunnett test to determine significant differences (C.I. 95%, $p < 0.05$) between sera of mice inoculated with the bacterins and the negative control. Unpaired Student *t* Test was used to determine significant difference between positive and negative swine sera.

## 3. Results

### 3.1. Recombinant antigen selection

According to the previously stipulated criteria, fifteen recombinant antigens were selected for this study (Table 1). The antigens MHP_0067, MHP_0223 and MHP_0596 do not have predicted adhesin activity. To confirm the antigenicity of these recombinant antigens, their reactivity with positive and negative swine sera was evaluated by ELISA. All recombinant antigens were strongly recognized by positive swine sera ($p < 0.05$) (Table 2), supporting our previous characterization demonstrating that recombinant antigens maintain reactive epitopes similar to those found in native proteins [25,26].