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Potential use of a recombinant replication-defective adenovirus vector carrying the C-terminal portion of the P97 adhesin protein as a vaccine against *Mycoplasma hyopneumoniae* in swine

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ABSTRACT

Mycoplasma hyopneumoniae causes severe economic losses to the swine industry worldwide and the prevention of its related disease, enzootic porcine pneumonia, remains a challenge. The P97 adhesin protein of *M. hyopneumoniae* should be a good candidate for the development of a subunit vaccine because antibodies produced against P97 could prevent the adhesion of the pathogen to the respiratory epithelial cells *in vitro*. In the present study, a P97 recombinant replication-defective adenovirus (rAdP97c) subunit vaccine efficiency was evaluated in pigs. The rAdP97c vaccine was found to induce both strong P97 specific humoral and cellular immune responses. The rAdP97c vaccinated pigs developed a lower amount of macroscopic lung lesions ($18.5 \pm 9.6\%$) compared to the unvaccinated and challenged animals ($45.8 \pm 11.5\%$). rAdP97c vaccine reduced significantly the severity of inflammatory response and the amount of *M. hyopneumoniae* in the respiratory tract. Furthermore, the average daily weight gain was slightly improved in the rAdP97c vaccinated pigs (0.672 ± 0.068 kg/day) compared to the unvaccinated and challenged animals (0.568 ± 0.104 kg/day). A bacterin-based commercial vaccine (Suvaxyn® MH-one) was more efficient to induce a protective immune response than rAdP97c even if it did not evoke a P97 specific immune response. These results suggest that immunodominant antigens other than P97 adhesin are also important in the induction of a protective immune response and should be taken into account in the future development of *M. hyopneumoniae* subunit vaccines.

1. Introduction

Mycoplasma hyopneumoniae is the etiological agent of enzootic porcine pneumonia (PEP), which is one of the most economically significant diseases found in the porcine industry worldwide [1]. The *M. hyopneumoniae* chronic infection is accompanied by non-productive coughing, retarded growth and inefficient food conversion [1]. *M. hyopneumoniae* colonizes the ciliated epithelial cells of the respiratory tract, thereby damaging them [2,3] and which predisposes infected animals to secondary invaders such as porcine reproductive and respiratory syndrome virus and *Actinobacillus pleuropneumoniae* [4,5]. *M. hyopneumoniae* infection also causes an intensive inflammatory immune response in the bronchus-associated lymphoid tissue (BALT) which contributes to damage the lungs [6,7].

Traditionally, PEP is controlled using vaccines combined with hygiene and management procedures. As for many infectious diseases, the vaccination remains an effective approach to prevent and eradicate PEP. The commonly used vaccines against *M. hyopneumoniae* are in the form of bacterins. Several studies have demonstrated that these vaccines have a partial protective effect since vaccinated animals stay infected for a long period of time. Consequently, vaccines fail to prevent the transmission of the pathogen to susceptible animals [8,9]. This partial protection could be due to the fact that bacterins are administered parenterally and, therefore, do not stimulate a strong mucosal immunity [10].

Current efforts to develop an effective vaccine against *M. hyopneumoniae* are shifted toward subunit-based vaccines. Some immunodominant antigens of the pathogen have been identified, and they include lipoproteins P65, Mhp378 and Mhp651 proteins [11,12], the cytolosic P36 protein [13], ribonucleotide reductase (nrdF) [14] and the P97 adhesin [15]. However, only the nrdF and P97 proteins have been experimentally tested as subunit vaccine candidates in pigs and were able to provide partial protection in

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vaccinated animals [16–18]. P97 fulfills important prerequisites for the development of an effective subunit vaccine. It is considered as a major adhesin of *M. hyopneumoniae* [15,19], infected pigs develop immune responses against P97 and the antibodies produced against this protein prevent the adhesion of the pathogen to the respiratory epithelial cells *in vitro*[15]. Moreover, the encoding P97 gene is found in all *M. hyopneumoniae* isolates tested so far [19,20]. Furthermore, P97 is well characterized. P97 contains two repeated regions, RR1 and RR2, located in the C-terminal portion [21]. The cilium binding sites are located in the RR1 region and at least seven AAKPV/E repeats are required for functional binding [22]. The RR2 region is involved in the attachment of *M. hyopneumoniae* to the extracellular matrix of the respiratory tract [23].

As *M. hyopneumoniae* infection is restricted to the respiratory tract, an ideal subunit vaccine should be mucosally administered and should evoke both local humoral and cell-mediated immune responses [16,17]. One of the hallmark of the mucosal immune system is the production of secretory IgA (sIgA) which can prevent infection and favor the removal of the pathogens [10]. Recombinant adenovirus vectors (rAd) have a natural tropism for cells of mucosa such as the porcine respiratory tract [24–26]. In addition, rAd are (1) able to deliver efficiently the recombinant transgene(s) to the antigen-presenting cells; (2) able to induce both humoral and cellular specific immune responses against the recombinant expressed proteins; (3) suitable for high-yield production *in vitro* [27,28]. Considering these advantages, a recombinant replication-defective adenovirus vector expressing the C-terminal portion (containing the regions RR1 and RR2) of the P97 adhesin (P97c), designated rAdP97c, was previously generated and its immunogenicity was tested in mice [29].

Therefore, the objectives of the present study were (i) to evaluate the capacity of rAdP97c to induce a P97c-specific immune response in pigs following mucosal administration, and (ii) to determine its protective efficacy in vaccinated pigs following a *M. hyopneumoniae* challenge infection.

2. Materials and methods

2.1. M. hyopneumoniae strains and proteins

The *M. hyopneumoniae* strain used for the challenge was the virulent strain 232. This strain was cultured in modified Friis medium containing 20% porcine serum, 5% yeast extract, 150 μg/mL bacitracin, 100 μg/mL ampicillin, 7.5 μg/mL colistin, 2.5 μg/mL amphotericin B and 40 μg/mL phenol red used as an indicator of growth. Bacteria were harvested and resuspended in phosphate buffered saline (PBS). The titer of *M. hyopneumoniae* was determined as described elsewhere [20], and expressed as color changing units (CCU) per mL. To extract total *M. hyopneumoniae* proteins, bacteria were resuspended in PBS containing 1 mM PMSF and 1 mM of pefabloc (Boehringer, Mannheim, Germany), and then lyzed on ice by sonication. The total protein extract was collected and kept at −80 °C until use.

The recombinant P97c protein (rP97c) was produced in *Escherichia coli* strain BL2(DE3)pLysS as previously described [29]. Protein concentrations were determined using the Bradford method (Bio-Rad, Mississauga, ON, Canada).

2.2. Vaccines

The rAd used in this study was a replicative-defective E1 and E3 deleted human type 5 (AdΔE1/E3). The rAdP97c vaccine designed to express the C-terminal portion of the *M. hyopneumoniae* P97 adhesin (P97c) of the 25934 strain (ATCC, Rockville, MD) was obtained from a previous study [29]. After amplification in 293 cells, the rAdP97c was purified by ultracentrifugation on double cesium chloride gradient, and frozen at −80 °C in PBS. The titer of rAdP97c was determined using the 50% tissue culture infectious dose ($TCID_{50}$) method. For comparison purposes, the commercially available bacterin-based vaccine, the Suvaxyn® MH-one (Wyeth Animal Health, Guelph, ON, Canada), was also included in this study.

2.3. Vaccination and infection of pigs

M. hyopneumoniae negative pigs (3 weeks old) were purchased from F. Menard Inc. (Ange-Gardien, Québec, Canada) and maintained in the animal facility of the Canadian Food Inspection Agency (St-Hyacinthe, Québec, Canada). All experiments were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care. A total of 28 pigs were included in the study and divided into four groups as follows: (a) control group (non-vaccinated and non-challenged animals, $n = 5$); (b) unvaccinated group (non-vaccinated and challenged animals, $n = 5$); (c) Suvaxyn® MH-one vaccinated group (animals were vaccinated once by intramuscular route, according to the manufacturer's instructions and challenged, $n = 8$); (d) rAdP97c vaccinated group (animals were vaccinated with 2×10^{10} $TCID_{50}$ of rAdP97c twice (at days 0 and 14) by intranasal (i.n.) route and challenged, $n = 10$). The challenge was performed at day 28 after the first vaccination with 10^6 CCU of the 232 *M. hyopneumoniae* strain by intratracheal route.

Animals were weighed at days 0, 28, 42 and 56 after the first vaccination (post-vaccination) to determine the average daily weight gain (ADG). All animals were euthanized at day 56 post-vaccination and lungs were removed to determine the percentage of macroscopic lung lesions as described elsewhere [30]. Sections of the lungs were also taken for microscopic examination and *M. hyopneumoniae* quantification.

2.4. Humoral immune response detection by ELISA

Sera were collected at days 0, 14, 28, 42 and 56 and saliva at days 28 and 56 post-vaccination. The HerdChek *M. hyopneumoniae* antibody ELISA kit (IDEXX Laboratories Inc., Westbrook, ME, USA) was performed to determine the serological status of pigs against *M. hyopneumoniae* antigens. According to the manufacturer's instructions, the tested samples are considered seropositive if the sample/positive control (S/P) ratio is higher than 0.3. For the detection of P97c-specific antibodies, polypropylene 96 wells plates (Nalge Numc International, Rochester, NY, USA) were coated with rP97c (0.5 μg/well) and incubated overnight at 4 °C. The plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked with 5% non-fat milk in PBS (PBSM). Following two wash steps with PBST, the plates were incubated with 100 μl of sera or saliva (diluted 1:200 in PBSM). Following three washes with PBST, the plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-porcine IgG or IgA (Bethyl Laboratories Inc., Montgomery, TX, USA). In order to detect IgG subclasses, the plates were first incubated with mouse anti-porcine IgG1 or IgG2a (Serotec, Kidlington, Oxford, England) before they were incubated with HRP-conjugated anti-mouse IgG heavy-plus-light-chain (Serotec). After washing, 100 μl/well of 3, 3′-5,5′-tetramethyl benzedine (TMB) substrate (Zymed, San Francisco, CA, USA) were added. The color reaction was stopped following the addition of 1N H_2SO_4 and absorbance was read at 450 nm with an ELISA plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). The antibody titers were determined by extrapolation from standard curves which were generated with a pool of serum or saliva from rAdP97c vaccinated pigs as previously described [31].Lymphocyte proliferation assay