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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Keywords: *Mycoplasma hyopneumoniae*, P97 adhesin, replication-defective adenovirus, vaccination, porcine enzootic pneumonia

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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% ± 9.6) compared to the unvaccinated and challenged animals (45.8% ± 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 kg/day ± 0.068) compared to the unvaccinated and challenged animals (0.568 kg/day ± 0.104). 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 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 repeat 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; and 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 1mM PMSF and 1mM 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); 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 X 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].

2.5. Lymphocyte proliferation assay

Blood samples were taken (at days 0, 14, 28, 42 and 56) in heparinized tubes (Monoject, Mansfield, MA, USA). The peripheral blood mononuclear cells (PBMC) were purified by density gradient centrifugation using Ficoll-Hypaque-1077 (Sigma-Aldrich, Oakville, ON, Canada) according to the manufacturer's instructions. The purified PBMC were resuspended at a concentration of 2 x 10^6 cells/mL in RPMI 1640 culture medium supplemented with 10% of fetal bovine serum (FBS), 2 mM glutamine, 50 µM ß-mercaptoethanol, and 100 U/mL penicillin/streptomycin. A 100 µl aliquot was dispatched into each well of a 96-well plate. One hundred µl of rP97c or total *M. hyopneumoniae* proteins (which both contain a total of 10 µg of protein/mL) were added to each well in triplicate. As a positive control, cells were also stimulated with Concanavalin (ConA) at a final concentration of 10 µg/mL. After 48 hrs incubation at 37°C with 5% CO_2, cells were treated with 10 µM BrdU (Exalpha Biologicals Inc, MA. USA) and incubated for an additional period of 16 hrs. The cell incorporation of BrdU was measured using the BrdU Cell Proliferation Assay Kit (Exalpha Biologicals) according to the manufacturer's instructions. The stimulation index (SI) was calculated by dividing the 450 nm absorbance results of the stimulated cells by the 450 nm absorbance results of the non-stimulated cells.

2.6. Cytological and cytokine analysis in the BALF

Bronchoalveolar lavage fluids (BALF) were collected at necropsy (day 56 after the first vaccination) as previously described [6]. Briefly, a volume of 30 mL of sterile PBS was infused into the lung using an 18-gauge catheter attached to a syringe. The BALF were

aspired back and passed through a single layer of gauze to remove gross mucus. Approximately 20 mL of the BALF was retrieved and immediately kept on ice.

The number of total leukocytes was determined manually using a hemacytometer. The differential cell counts of leukocytes were made using the Shandon cytospin 4 machine (Shandon Inc., Pittsburgh, Pa, USA). The cells were stained by cytocentrifugation using the Diff-Quick staining method. For each sample, 400 to 500 cells/cytospin were counted under the microscope.

Cytokines (IL-1β, IL-6, IL-8 and TNF-α) were measured in the BALF by sandwich ELISA using porcine-specific pair-matched antibodies from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's recommendations. The concentration was determined after extrapolation from standard curves prepared using the related purified cytokines supplied by the manufacturer. All assays were carried out in duplicate.

2.7. Quantification of *M. hyopneumoniae* in lungs and BALF

In order to determine the amount of *M. hyopneumoniae*, sections of lungs were taken from individual pigs and were homogenized using the Mini-Beadbeater^{TM} homogenizer (Biospec products, Bartlesville, OK, USA). Homogenates and BALF, were clarified by centrifugation. Then, supernatants were collected and diluted serially from 10^1 to 10^{10} in the growth medium of *M. hyopneumoniae*. For each dilution, a 200 µl aliquot was poured into 96 well microplates in triplicate. The plates were covered with an adhesive film and incubated at 37°C for 5 days. The amount of *M. hyopneumoniae* corresponds to the lower dilution where the color of medium changed from red to yellow and was expressed as CCU/mL.

2.8. In vitro *M. hyopneumoniae* growth inhibition assay

The *M. hyopneumoniae* growth inhibition assay was performed as described previously with slight modifications [29]. Briefly, 100 µl aliquots containing 10^5 CCU of the 232 strain of *M. hyopenumoniae* were poured into 96 well microplates. One hundred µl of serial dilutions (1:40 to 1:1280) of serum samples (from day 28 post-vaccination) were then added into wells in triplicate. As positive controls, mycoplasma was cultured in the absence of serum. Wells containing only the culture medium served as negative controls. After incubation at 37°C for 4 days, absorbance was recorded at 560 nm. The percentage of growth inhibition was determined as follows: [sample absorbance - absorbance of the positive control) / (negative control absorbance - absorbance of the positive control)] x 100.

2.9. Statistical analysis

The statistical analyses were realized using the GraphPad Prism version 4 software. The paired t test was used to establish the differences between the level of IgG1 and IgG2a antibodies in pig sera and saliva of each experimental group. The one-way ANOVA combined with the Bonferroni post-tests models was used to determine if there is statistical significant difference in regards to the percentage of macroscopic lung lesions between experimental groups. The regular two-way ANOVA combined with the Bonferroni post-tests models was used in all others statistical analyses. Values of $P < 0.05$ were considered to be significant.

3. RESULTS

3.1. Humoral immunity in rAdP97c vaccinated pigs

M. hyopneumoniae specific antibody response was analyzed by HerdChek *M. hyopneumoniae* Antibody ELISA kit ELISA from serum samples taken at day 0 (preimmune serum), 14, 28, 42 and 56 post-vaccination. As shown in Table 1, all pigs were negative at day 0, indicating that they were seronegative for the pathogen prior to the vaccination. The Suvaxyn® MH-one vaccinated animals showed a positive anti-*M. hyopneumoniae* antibody response before challenge. The unvaccinated pigs seroconverted following the challenge. In contrast, no antibody response could be detected neither in rAdP97c vaccinated nor non-vaccinated and non-challenged animals.

Significant levels of P97c-specific IgG and IgA were detected in sera of rAdP97c vaccinated pigs as early as day 14 post-vaccination (Fig. 1). P97c specific antibodies were also detected in unvaccinated animals after challenge, which indicates that the P97 adhesin was expressed *in vivo* during *M. hyopneumoniae* infection. The rAdP97c vaccinated pigs had significant levels of P97c-specific IgG and IgA (Fig. 1) in their saliva prior and after the challenge. No P97c specific antibody response was detected in the saliva of non-vaccinated and Suvaxyn® MH-one vaccinated animals prior to challenge but IgG and IgA were detected at 28 days post-challenge. Moreover, the level of saliva P97c specific IgA in Suvaxyn® MH-one vaccinated animals was significantly lower compared to rAdP97c vaccinated animals at 28 days post-challenge ($P < 0.001$). Both IgG subclasses were produced in sera and saliva of rAdP97c vaccinated animals, where a higher amount of IgG2a were produced compared to IgG1 ($P < 0.05$), suggesting a Th1-biased immune response (Fig. 2). Both IgG1 and IgG2a

P97c specific antibodies were found in unvaccinated and Suvaxyn® MH-one vaccinated animals only after challenge, with significantly higher amount of IgG1 compared to IgG2a ($P < 0.001$ and $P < 0.01$, respectively), indicating a Th2-biased immune response.

3.2. Cell-mediated immune response following rAdP97c vaccination

As illustrated in Fig. 3, only the rAdP97c vaccinated pigs PBMC, proliferated significantly in the presence of rP97c and the proliferation response was observed as early as day 14 post-vaccination ($P < 0.001$ at days 14, 28 and 42) and declined after the challenge ($P < 0.05$ at day 56). In contrast, significant SI were observed in the Suvaxyn® MH-one vaccinated animals only in the presence of *M. hyopneumoniae* cell lysate total proteins (Fig. 3). The proliferation response in this group decreased after the challenge to a point that at day 56 post-vaccination there was no statistical difference with the control group ($P > 0.05$). Interestingly, upon stimulation with ConA, no difference between the SI could be observed in all experimental groups before the challenge (days 14 and 28). However after the challenge, the SI of all challenged groups progressively decreased (Fig. 3). Taken together, these observations indicate that both rAdP97c and Suvaxyn® MH-one have the capacity to induce a cell-mediated immune response and that *M. hyopneumoniae* have the ability to induce an immunosuppression in infected animals which reduces the PBMC specific lymphocyte proliferation response against rP97c and *M. hyopneumoniae* cell lysate total proteins.

3.3. *M. hyopneumoniae* growth inhibition *in vitro*

As shown in Fig. 4, sera from rAdP97c vaccinated pigs inhibited the growth of mycoplasma cells in a dose dependent manner. A significant mycoplasma growth inhibitory effect could also be observed within sera from animals vaccinated with Suvaxyn® MH-one and was higher compared to rAdP97c vaccinated animals. As expected, no mycoplasma growth inhibitory activity was observed within sera of non-vaccinated animals.

3.4. Bacteriological and pneumonic findings

One week after the challenge, a non-productive coughing was observed in all challenged groups with a lesser extend in Suvaxyn® MH-one vaccinated animals (data not shown). No macroscopic lung lesions were observed in the control animals group. In contrast, the pneumonic lesions characteristics of PEP were present in all infected animals (Fig. 5). The unvaccinated and infected pigs had the higher percentage of pneumonic lesions (45.8% ± 11.5). In comparison to this group, the pigs vaccinated with rAdP97c and Suvaxyn® MH-one had significantly lower percentage of pneumonic lesions with means of 18.5% ± 9.6 and 1.3% ± 1.7, respectively. No statistical difference was found between the uninfected animals and Suvaxyn® MH-one vaccinated and challenged animals. Noteworthy, a significant difference was observed between the infected and rAdP97c vaccinated animals compared to uninfected animals. After examining the microscopic lung lesions by hematoxylin-oesine staining, the presence of nodular lymphoid hyperplasia was observed around the bronchioles in the lung tissues from all challenged groups (data not shown). As expected no *M. hyopneumoniae* was recovered in the samples from the control group. In contrast, *M. hyopneumoniae* was retrieved in all challenged animals, but significantly at

lower amount in rAdP97c and Suvaxyn® MH-one vaccinated animals compared to unvaccinated animals ($P < 0.05$ and $P < 0.001$, respectively) (Fig. 5). The amount of *M. hyopneumoniae* retrieved in BALF and lungs from Suvaxyn® MH-one vaccinated animals was significantly lower than in rAdP97c vaccinated animals ($P < 0.001$ and $P < 0.01$, respectively). Overall, the amount of *M. hyopneumoniae* retrieved from lungs of infected animals was higher compared to BALF ($P < 0.001$).

3.5. Growth performance of rAdP97c vaccinated pigs

As illustrated in Fig. 5, means ADG were not significantly different between the experimental groups before challenge. However, unvaccinated and rAdP97c vaccinated challenge animals displayed a significant retarded growth at day 42 post-vaccination compared to the control group ($P < 0.01$). At 42 days post-vaccination, no significant difference could be observed with Suvaxyn® MH-one vaccinated animals compared to the control, the unvaccinated and the rAdP97c vaccinated challenge groups. Furthermore, at the end of the experiment (day 56 post-vaccination), there was no statistical difference with the ADG values of Suvaxyn® MH-one vaccinated and infected animals (ADG = 0.743 ± 0.088 kg/day) compared to the control (ADG = 0.830 ± 0.037 kg/day) and rAdP97c vaccinated challenge animals (ADG = 0.657 ± 0.079 kg/day). There was no statistical difference in the ADG values between the unvaccinated and rAdP97c vaccinated challenge animals during the course of the experiment but there were significant differences when they were compared to control animals at days 42 and 56 post-vaccination. Nonetheless, the mean ADG value of rAdP97c vaccinated animals was slightly higher compared to unvaccinated animals with the consequence that Suvaxyn® MH-one vaccinated animals ADG value was statistically

different from unvaccinated animals ($P < 0.01$) but was not statistically different from rAdP97c vaccinated animals (Fig. 5). Overall, these results suggest that rAdP97c vaccine has a beneficial effect on the growth performance of pigs but at a lower extend than Suvaxyn® MH-one vaccine.

3.6. Inflammatory responses

As illustrated in Fig. 6, significant increases of total leukocytes were observed in unvaccinated (401.3 X 10^4 ± 187.0 cells/mL) and rAdP97c vaccinated challenge animals (253.7 X 10^4 ± 79.3 cells/mL) compared to the control group (19.5 X 10^4 ± 2.1 cells/mL). These increases were in average 20-fold for the unvaccinated group, 13-fold for rAdP97c vaccinated challenge animals. There was a significant increase of neutrophils in the BALF from unvaccinated (264.0 X 10^4 ± 68.9 cells/mL) and rAdP97c vaccinated challenge animals (158.6 X 10^4 ± 82.5 cells/mL) compared to the control group (0.570 X 10^4 ± 0.212 cells/mL). These latest results indicate that *M. hyopneumoniae* infection causes an important inflammatory immune response in the lungs. In addition, even if rAdP97c has a tendency to reduce the migration of neutrophils into the *M. hyopneumoniae* infected lungs compared to unvaccinated animals (only if the mean values are compared), it was not able to prevent the inflammatory immune response efficiently compared to Suvaxyn® MH-one commercial vaccine. Furthermore, in comparison to the control group and the Suvaxyn® MH-one vaccinated challenge animals, significant levels of IL-6 and IL-8 were detected in unvaccinated and rAdP97c vaccinated challenge animals ($P < 0.001$) (Fig. 6). In addition, no significant differences were observed in the levels of IL-1β, IL-6, IL-8 and TNFα with the uninfected and unvaccinated animals (control group) compared to Suvaxyn® MH-one

vaccinated challenge animals which clearly support the fact that this commercial vaccine efficiently prevented the inflammatory immune response. Noteworthy, the rAdP97c vaccination was able to reduce the IL-8 expression in infected animals compared to unvaccinated challenge animals ($P < 0.001$) (Fig. 6).

4. DISCUSSION

M. hyopneumoniae is the causative agent of PEP, which is one of the most contagious diseases in the swine industry [1]. As for many infectious diseases, vaccination remains the effective approach for preventing and eradicating PEP. Extensive efforts are currently oriented toward the development of subunit vaccines to address the shortcomings associated with the current bacterin-based commercial vaccines [16-18]. Herein, a replication-defective recombinant adenovirus-based vaccine encoding the C-terminal portion of the *M. hyopneumoniae* P97 adhesin protein (rAdP97c) was developed. The C-terminal portion of the P97 adhesin contains two regions called RR1 and RR2 that allow the adherence of the pathogen to host cells [22, 23]. The rAdP97c vaccine was assessed (i) for its capacity to induce a P97c-specific immune response in pigs, and (ii) for its protective efficacy in vaccinated pigs in a *M. hyopneumoniae* challenge trial. A bacterin-based commercial vaccine, the Suvaxyn® MH-one, was also included in the study as a vaccine control.

In the present study, it was found that rAdP97c i.n. inoculation was able to induce a specific mucosal as well as systemic immune responses in pigs against P97c recombinant protein which was characterized by the production of high levels of P97c specific IgG (with the IgG2a response dominated over the IgG1 response) and IgA antibodies in the sera and saliva of vaccinated animals, even before *M. hyopneumoniae* challenge (Fig. 1). Previous studies have also demonstrated that i.n. immunization with rAd vectors stimulates both mucosal and systemic antibody responses to the encoded antigen and preferentially promotes a Th1-type immune response [32-34]. It is not known whether the pigs have a Th1/Th2 system similar to the one described in mice [35]. Nevertheless, it is tempting to speculate that

immunization with rAdP97c triggers a substantial P97c-specific Th1-type response and a moderate Th2-type response in vaccinated pigs (Fig. 2).

The actual contribution of both types of immune response in the protection against *M. hyopneumoniae* infections is not clearly defined. The first vaccination trial using a recombinant P97 protein administered intramuscularly failed to protect the vaccinated pigs against *M. hyopneumoniae* [36]. Shimoji et al. evaluated *Erysipelothrix rhusiopathiae* YS-19 expressing the C-terminal portion of P97 as an i.n. administered vaccine against *M. hyopneumoniae.* They observed a significant reduction of macroscopic pneumonic lesions despite the fact that this vaccine failed to induce detectable antibodies against P97 [17]. On the other hand, Thacker et al. have tested different bacterin-based vaccines against *M. hyopneumoniae*, and found that the humoral immunity appeared to correlate substantially with the reduction of the percentage of pneumonic lesions compared to the cell-mediated immunity [37]. It has been reported that local sIgA prevents the adhesion of mycoplasmas to host cells, and IgG enhances their phagocytosis and opsonization [38, 39]. In a previous study, it was shown that sera of rAdP97c immunized mice were able to prevent the growth of *M. hyopneumoniae in vitro* but not sera of nrAd immunized mice [29] which have suggested that specific P97c antibodies could be involved in that phenomenon. Herein, sera of rAdP97c and Suvaxyn® MH-one vaccinated pigs were also able to inhibit the growth of *M. hyopneumoniae in vitro* (Fig. 4). A specific cell-mediated immune response against P97c was also detected in PBMC of rAdP97 vaccinated pigs but not in PBMC of Suvaxyn® MH-one vaccinated pigs (Fig. 3). Given the propensity of *M. hyopneumoniae* for the suppression of cell-mediated immune response [16, 18, 40], it was not suprising that PBMC from the *M hyopneumoniae* infected animals showed a decreased SI when stimulated *in vitro* with ConA

and all tested antigens (rP97c and *M hyopneumoniae* cell lysate total protein) (Fig. 3). Noteworthy, the mechanism by which the pathogen induces the PBMC cell-mediated immunosuppression is not known. Since the immunosuppressive effect was observed in PBMCs, this may be due mainly to the regulation of the immune response of the host rather than a direct effect of the bacterium [41].

To evaluate whether P97c-specific immune response was protective, the vaccinated pigs were challenged. Interestingly, the rAdP97c vaccination had a positive effect against *M. hyopneumoniae* challenge by lowering the amount of *M. hyopneumoniae* recovered from tissues (Fig. 5). There was a significant reduction of *M. hyopneumoniae* amount recovered from the respiratory tract (in lungs and BALF) and a significant reduction of the percentage of macroscopic lung lesions in rAdP97c vaccinated animals (Fig. 5). Furthermore, the rAdP97c vaccinated group also had an average ADG higher than the unvaccinated group, although there was no statistical difference between both groups. In addition, more neutrophils were detected in the BALF of the unvaccinated challenge group than in the rAdP97c vaccinated and challenge group (Fig. 6), indicating that inflammatory response was milder in animals vaccinated with rAdP97c. IL-8 acts primarily on the activation and migration of neutrophils [42]. Therefore, it was not surprising that BALF of the unvaccinated challenge animals showed a higher concentration of IL-8 than rAdP97c vaccinated animals (Fig. 6). *M. hyopneumoniae* infection is well known to increase cytokines expression, such as IL-1β, IL-6, IL-8 and TNF-α [43]. In the previous published reports, several approaches were used to detect those cytokine expressions such as bioassay, qPCR for mRNA expression and ELISA. If we take into account the technique that was presently used (ELISA), the type of sample tested (BALF) and the time post-infection (28 days post-infection), the level of IL-

1β, IL-6, IL-8 and TNF-α expression detected in BALF of *M hyopneumoniae* infected animals is in accordance with a previous report published by Thanawongnuwech et al. (2004).

For all protection criteria evaluated in the present study, rAdP97c was less efficient than the Suvaxyn® MH-one vaccine. The fact that Suvaxyn® MH-one vaccine contains several immunodominant antigens of *M. hyopneumoniae* may explain its superiority over the rAd97c subunit vaccine but at least P97c protein alone is able to induce a partial protective immune response by reducing 1) the macroscopic lung lesions; 2) the amount of *M. hyopneumoniae* recovered from tissues and 3) the local inflammation. On the other hand, the *M. hyopneumoniae* challenge strain used, the heterologous 232 strain, may also have an effect on the efficacy of rAdP97c. Indeed, the homologous ATCC 25934 (where the P97c gene originate) and the 232 strain differ in the number of the AAKPV/E repeat sequence: 15 for the 232 strain and 10 for the ATCC 25934 strain (data not shown). From the obtained results rise several questions. 1) Is it possible to generate a very efficient vaccine against *M. hyopneumoniae* with a subunit vaccine comprised of a single antigen? 2) What is the importance of the antigenic variations including that of the P97 adhesin in regards to the protective immune response?

In conclusion, our results demonstrate that the rAdP97c vaccine induces high P97c specific cell-mediated and humoral immune responses and partially protects the pigs against *M. hyopneumoniae* challenge. However, further studies are necessary to improve this subunit vaccine approach which should involve the inclusion of other *M. hyopneumoniae* antigens in addition to the P97c protein.

ACKNOWLEDGMENTS

The authors are grateful to Dr Eileen L. Thacker (College of Veterinary Medicine, Iowa State University, USA) for kindly providing the 232 *M. hyopneumoniae* pathogenic strain. The authors are grateful to Dr Johanne Elsener (Wyeth Animal Health, Guelph, Ontario, Canada) for kindly providing the Suvaxyn®MH-one vaccine. The authors are also grateful to Cynthia M. Guilbert for critically reviewing the manuscript. This work was supported financially by the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ). The authors would like to thank in particular the decease Dr Serge Dea (INRS-Institut Armand-Frappier) for his initial contribution to the project.

REFERENCES

[1] Thacker EL. Mycoplasmal diseases. In: Straw BE, Zimmermann J, D'Allaire S, Taylor DJ, editors, Diseases of swine 9th ed Ames: Iowa State University Press 2006:p.700-13.

[2] DeBey MC, Ross RF. Ciliostasis and loss of cilia induced by Mycoplasma hyopneumoniae in porcine tracheal organ cultures. Infect Immun 1994 Dec;62(12):5312-8.

[3] Zielinski GC, Ross RF. Adherence of Mycoplasma hyopneumoniae to porcine ciliated respiratory tract cells. Am J Vet Res 1993 Aug;54(8):1262-9.

[4] Thacker EL, Halbur PG, Ross RF, Thanawongnuwech R, Thacker BJ. Mycoplasma hyopneumoniae potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. J Clin Microbiol 1999 Mar;37(3):620-7.

[5] Marois C, Dory D, Fablet C, Madec F, Kobisch M. Development of a quantitative Real-Time TaqMan PCR assay for determination of the minimal dose of Mycoplasma hyopneumoniae strain 116 required to induce pneumonia in SPF pigs. J Appl Microbiol 2009 Oct 7.

[6] Okada M, Asai T, Ono M, Sakano T, Sato S. Cytological and immunological changes in bronchoalveolar lavage fluid and histological observation of lung lesions in pigs immunized with Mycoplasma hyopneumoniae inactivated vaccine prepared from broth culture supernate. Vaccine 2000 Jun 15;18(25):2825-31.

[7] Lorenzo H, Quesada O, Assuncao P, Castro A, Rodriguez F. Cytokine expression in porcine lungs experimentally infected with Mycoplasma hyopneumoniae. Vet Immunol Immunopathol 2006 Feb 15;109(3-4):199-207.

[8] Haesebrouck F, Pasmans F, Chiers K, Maes D, Ducatelle R, Decostere A. Efficacy of vaccines against bacterial diseases in swine: what can we expect? Vet Microbiol 2004 Jun 3;100(3-4):255-68.

[9] Maes D, Segales J, Meyns T, Sibila M, Pieters M, Haesebrouck F. Control of Mycoplasma hyopneumoniae infections in pigs. Vet Microbiol 2008 Jan 25;126(4):297-309.

[10] Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 2006 Feb;6(2):148-58.

[11] Kim MF, Heidari MB, Stull SJ, McIntosh MA, Wise KS. Identification and mapping of an immunogenic region of Mycoplasma hyopneumoniae p65 surface lipoprotein expressed in Escherichia coli from a cloned genomic fragment. Infect Immun 1990 Aug;58(8):2637-43.

[12] Meens J, Selke M, Gerlach GF. Identification and immunological characterization of conserved Mycoplasma hyopneumoniae lipoproteins Mhp378 and Mhp651. Vet Microbiol 2006 Aug 25;116(1-3):85-95.

[13] Strasser M, Frey J, Bestetti G, Kobisch M, Nicolet J. Cloning and expression of a species-specific early immunogenic 36-kilodalton protein of Mycoplasma hyopneumoniae in Escherichia coli. Infect Immun 1991 Apr;59(4):1217-22.

[14] Fagan PK, Djordjevic SP, Eamens GJ, Chin J, Walker MJ. Molecular characterization of a ribonucleotide reductase (nrdF) gene fragment of Mycoplasma hyopneumoniae and assessment of the recombinant product as an experimental vaccine for enzootic pneumonia. Infect Immun 1996 Mar;64(3):1060-4.

[15] Zhang Q, Young TF, Ross RF. Identification and characterization of a Mycoplasma hyopneumoniae adhesin. Infect Immun 1995 Mar;63(3):1013-9.

[16] Fagan PK, Walker MJ, Chin J, Eamens GJ, Djordjevic SP. Oral immunization of swine with attenuated Salmonella typhimurium aroA SL3261 expressing a recombinant antigen of Mycoplasma hyopneumoniae (NrdF) primes the immune system for a NrdF specific secretory IgA response in the lungs. Microb Pathog 2001 Feb;30(2):101-10.

[17] Shimoji Y, Oishi E, Muneta Y, Nosaka H, Mori Y. Vaccine efficacy of the attenuated Erysipelothrix rhusiopathiae YS-19 expressing a recombinant protein of Mycoplasma hyopneumoniae P97 adhesin against mycoplasmal pneumonia of swine. Vaccine 2003 Jan 17;21(5-6):532-7.

[18] Ogawa Y, Oishi E, Muneta Y, Sano A, Hikono H, Shibahara T, et al. Oral vaccination against mycoplasmal pneumonia of swine using a live Erysipelothrix rhusiopathiae vaccine strain as a vector. Vaccine 2009 Jul 16;27(33):4543-50.

[19] Wilton JL, Scarman AL, Walker MJ, Djordjevic SP. Reiterated repeat region variability in the ciliary adhesin gene of Mycoplasma hyopneumoniae. Microbiology 1998 Jul;144 (Pt 7):1931-43.

[20] Meyns T, Maes D, Calus D, Ribbens S, Dewulf J, Chiers K, et al. Interactions of highly and low virulent Mycoplasma hyopneumoniae isolates with the respiratory tract of pigs. Vet Microbiol 2007 Feb 25;120(1-2):87-95.

[21] Hsu T, Minion FC. Identification of the cilium binding epitope of the Mycoplasma hyopneumoniae P97 adhesin. Infect Immun 1998 Oct;66(10):4762-6.

[22] Minion FC, Adams C, Hsu T. R1 region of P97 mediates adherence of Mycoplasma hyopneumoniae to swine cilia. Infect Immun 2000 May;68(5):3056-60.

[23] Jenkins C, Wilton JL, Minion FC, Falconer L, Walker MJ, Djordjevic SP. Two domains within the Mycoplasma hyopneumoniae cilium adhesin bind heparin. Infect Immun 2006 Jan;74(1):481-7.

[24] Papp Z, Babiuk LA, Baca-Estrada ME. Induction of immunity in the respiratory tract and protection from bovine herpesvirus type 1 infection by different routes of immunization with recombinant adenovirus. Viral Immunol 1998;11(2):79-91.

[25] Torres JM, Sanchez C, Sune C, Smerdou C, Prevec L, Graham F, et al. Induction of antibodies protecting against transmissible gastroenteritis coronavirus (TGEV) by recombinant adenovirus expressing TGEV spike protein. Virology 1995 Nov 10;213(2):503-16.

[26] Rosenthal KL, Copeland KFT, Gallichan WS. Recombinant adenoviruses as vectors for mucosal immunity. In: Press A, editor. Mucosal Vaccines. San Diego, 1996: 147-58.

[27] Graham FL. Growth of 293 cells in suspension culture. J Gen Virol 1987 Mar;68 (Pt 3):937-40.

[28] Fallaux FJ, Bout A, van der Velde I, van den Wollenberg DJ, Hehir KM, Keegan J, et al. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. Hum Gene Ther 1998 Sep 1;9(13):1909-17.

[29] Okamba FR, Moreau E, Cheikh Saad Bouh K, Gagnon CA, Massie B, Arella M. Immune responses induced by replication-defective adenovirus expressing the C-terminal portion of the Mycoplasma hyopneumoniae P97 adhesin. Clin Vaccine Immunol 2007 Jun;14(6):767-74.

[30] Sorensen V, Jorsal SV, Mousing J. Diseases of the respiratory system. In: Straw BE, Zimmermann J, D'Allaire S, Taylor DJ, editors, Diseases of swine 9th ed Ames: Iowa State University Press 2006:p. 149-77.

[31] Mattoo S, Miller JF, Cotter PA. Role of Bordetella bronchiseptica fimbriae in tracheal colonization and development of a humoral immune response. Infect Immun 2000 Apr;68(4):2024-33.

[32] Guo L, Wang J, Zhou H, Si H, Wang M, Song J, et al. Intranasal administration of a recombinant adenovirus expressing the norovirus capsid protein stimulates specific humoral, mucosal, and cellular immune responses in mice. Vaccine 2008 Jan 24;26(4):460-8.

[33] Lemiale F, Kong WP, Akyurek LM, Ling X, Huang Y, Chakrabarti BK, et al. Enhanced mucosal immunoglobulin A response of intranasal adenoviral vector human immunodeficiency virus vaccine and localization in the central nervous system. J Virol 2003 Sep;77(18):10078-87.

[34] Xiang Z, Li Y, Gao G, Wilson JM, Ertl HC. Mucosally delivered E1-deleted adenoviral vaccine carriers induce transgene product-specific antibody responses in neonatal mice. J Immunol 2003 Oct 15;171(8):4287-93.

[35] Stevens TL, Bossie A, Sanders VM, Fernandez-Botran R, Coffman RL, Mosmann TR, et al. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature 1988 Jul 21;334(6179):255-8.

[36] King KW, Faulds DH, Rosey EL, Yancey RJ, Jr. Characterization of the gene encoding Mhp1 from Mycoplasma hyopneumoniae and examination of Mhp1's vaccine potential. Vaccine 1997 Jan;15(1):25-35.

[37] Thacker EL, Thacker BJ, Boettcher TB, Jayappa H. Comparison of antibody production, lymphocyte stimulation, and protection induced by four commercial *Mycoplasma hyopneumoniae* bacterins. Swine Health Prod 1998;6:107-12.

[38] Sheldrake RF, Romalis LF, Saunders MM. Serum and mucosal antibody responses against Mycoplasma hyopneumoniae following intraperitoneal vaccination and challenge of pigs with M hyopneumoniae. Res Vet Sci 1993 Nov;55(3):371-6.

[39] Sarradell J, Andrada M, Ramirez AS, Fernandez A, Gomez-Villamandos JC, Jover A, et al. A morphologic and immunohistochemical study of the bronchus-associated lymphoid

tissue of pigs naturally infected with Mycoplasma hyopneumoniae. Vet Pathol 2003 Jul;40(4):395-404.

[40] Kishima M, Ross RF. Suppressive effect of nonviable Mycoplasma hyopneumoniae on phytohemagglutinin-induced transformation of swine lymphocytes. Am J Vet Res 1985 Nov;46(11):2366-8.

[41] Vanden Bush TJ, Rosenbusch RF. Characterization of the immune response to Mycoplasma bovis lung infection. Vet Immunol Immunopathol 2003 Jul 15;94(1-2):23-33.

[42] Hechtman DH, Cybulsky MI, Fuchs HJ, Baker JB, Gimbrone MA, Jr. Intravascular IL-8. Inhibitor of polymorphonuclear leukocyte accumulation at sites of acute inflammation. J Immunol 1991 Aug 1;147(3):883-92.

[43] Thanawongnuwech R, Thacker B, Halbur P, Thacker EL. Increased production of proinflammatory cytokines following infection with porcine reproductive and respiratory syndrome virus and Mycoplasma hyopneumoniae. Clin Diagn Lab Immunol 2004 Sep;11(5):901-8.

Table 1. HerdChek *M. hyopneumoniae* Antibody ELISA assay antibodies detection results.

	Sample/positive control (SP) ratio ± standard deviation (SD)[a]				
Experimental groups	**Day 0**	**Day 14**	**Day 28**	**Day 42**	**Day 56**
Control (n = 5)	0.013 ± 0.015	0.026± 0.057	0,042 ± 0,027	0.044 ± 0.021	0,062 ± 0,014
Unvaccinated (n = 5)	0,028 ± 0,025	0.028 ± 0.024	0.022 ± 0,019	0.194 ± 0.083	0.36 ± 0,182
Suvaxyn® MH-one (n =8)	0,029 ± 0,024	0,24 ± 0.019	0,455 ± 0,164	1,3 ± 0,426	0.75 ± 0,501
rAdP97c (n = 10)	0,026 ± 0,013	0.044 ± 0.104	0,055 ± 0,085	0.154 ± 0,094	0.096 ± 0.084

The pigs were vaccinated with rAdP97c (at days 0 and 14) or Suvaxyn® MH-one (at day 0), and challenged with the virulent *M. hyopneumoniae* 232 strain at day 28 post-vaccination (after the first vaccination). Sera were collected at days 0, 14, 28, 42 and 56 post-vaccination and examined for the presence of antibodies against *M. hyopneumoniae.*

[a] seropositive if the S/P ratio is greater than 0.3.

FIGURE LEGENDS

Fig.1. P97c-specific IgG and IgA antibodies production in vaccinated pigs. Pigs were unvaccinated or vaccinated with rAdP97c (at days 0 and 14) or Suvaxyn® MH-one (at day 0) and challenged with the virulent *M. hyopneumoniae* 232 strain at day 28 post-vaccination. ELISA assays were performed using the rP97c as an antigen with sera and saliva. When 2 sets of data are labeled with superscripts of different letters or when only one set is labeled with a superscript, it indicates that these 2 sets of data are statistically different ($P < 0.05$).

Fig.2. P97c specific IgG1 and IgG2a antibodies production in vaccinated pigs. ELISA assays were performed using rP97c as an antigen and with sera and saliva. Experimental groups: unvaccinated and uninfected control animals (Ctl); unvaccinated and infected animals (Unvac); Suvaxyn® MH-one vaccinated and infected animals (MH-one); rAdP97c vaccinated and infected animals (rAdP97c). *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$ (significant difference between IgG1 and IgG2a).

Fig. 3. P97c specific lymphocyte proliferation immune responses. The PBMC were stimulated with rP97c or with the total mycoplasma cell lysate proteins or with Concanavalin A. The lymphocyte proliferation responses were measured as described in materials and methods. Data are expressed as stimulation index (SI) which represent the ratio of the BrdU cell incorporation of the stimulated cells divided by the BrdU cell incorporation of the non-stimulated cells (see materials and methods section). When 2

sets of data are labeled with superscripts of different letters or when only one set is labeled with a superscript, it indicates that these 2 sets of data are statistically different ($P < 0.05$).

Fig. 4. Growth inhibition of *M. hyopneumoniae* by sera of vaccinated pigs. Mycoplasma cells (10^5 CCU) were cultured as described in materials and methods in the presence of serial dilutions of sera collected at day 28 post-vaccination. The percentage of growth inhibition was determined as follows: [absorbance sample - absorbance of positive control) / (absorbance of negative control - absorbance positive control)] x 100.

Fig. 5. Level of protection induced in rAdP97c vaccinated pigs. Experimental groups: unvaccinated and uninfected control animals (Ctl); unvaccinated and infected animals (Unvac); Suvaxyn® MH-one vaccinated and infected animals (MH-one); rAdP97c vaccinated and infected animals (rAdP97c). BALF: bronchoalveolar lavage fluid; CCU: color changing unit; ADG: average daily weight gain. When 2 sets of data are labeled with superscripts of different letters or when only one set is labeled with a superscript, it indicates that these 2 sets of data are statistically different ($P < 0.05$).

Fig. 6. Inflammatory immune responses in the bronchoalveolar lavage fluids. BALF were collected at necropsy as described in the materials and methods section. Macrophages (Mac); neutrophils (Neu); lymphocytes (Lym). When 2 sets of data are labeled with superscripts of different letters or when only one set is labeled with a superscript, it indicates that these 2 sets of data are statistically different ($P < 0.05$).

Figure 1
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Figure 1. P97c specific IgG and IgA antibodies in vaccinated pigs

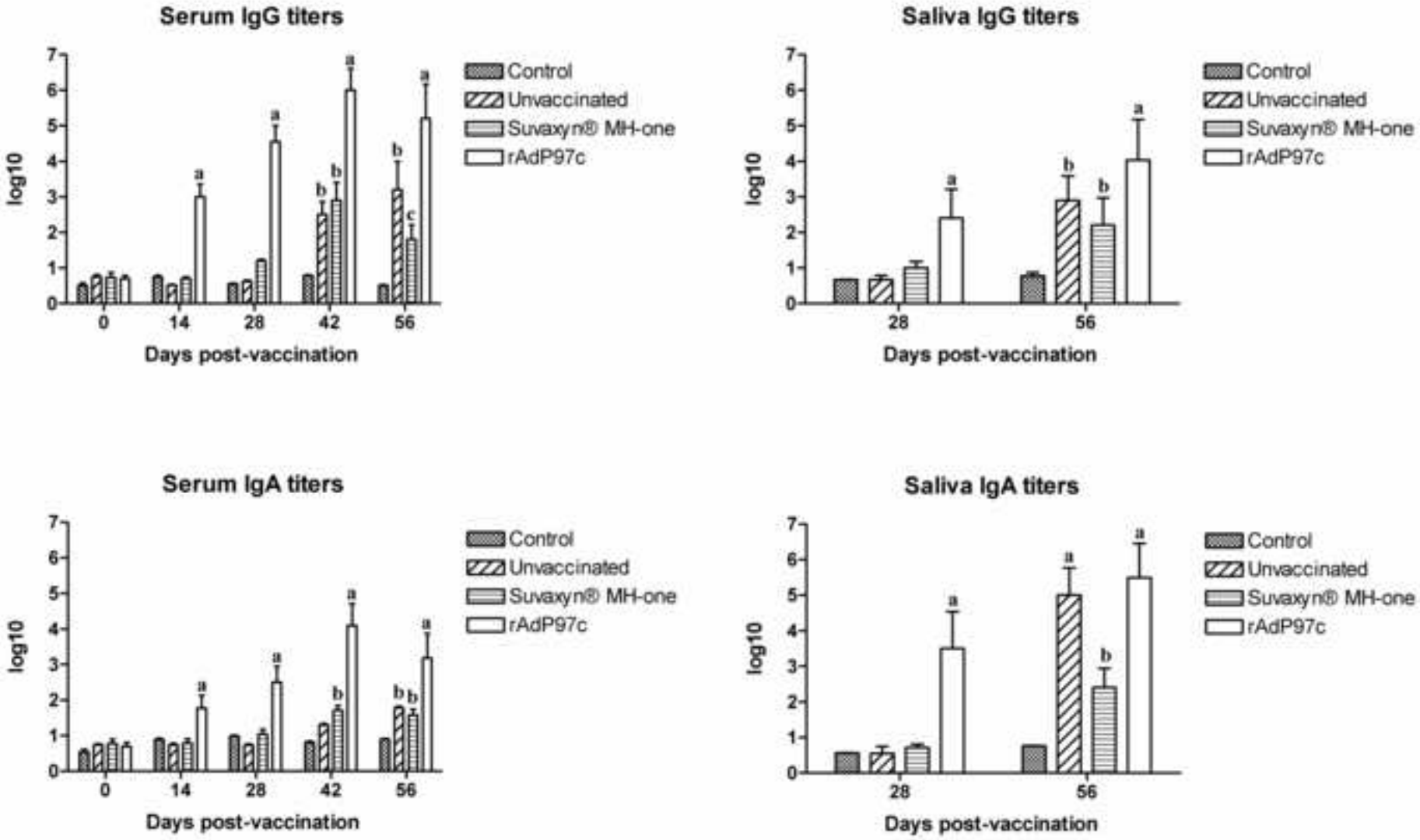

Figure 2
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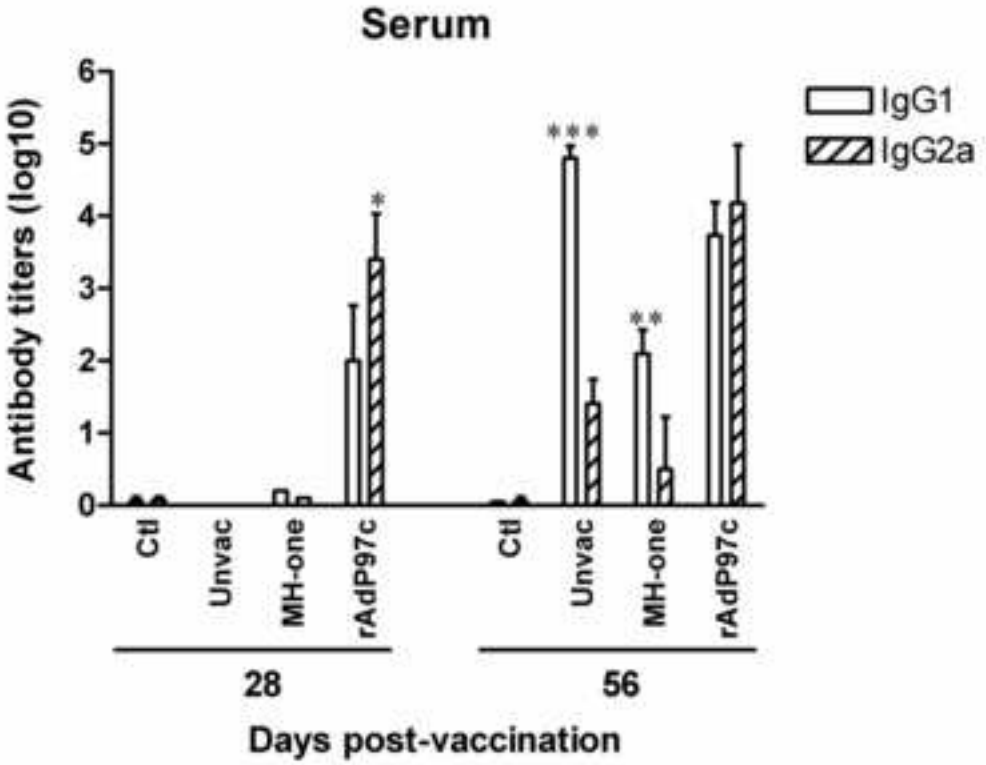
Figure 2. P97c specific IgG1 and IgG2a antibodies in vaccinated pigs.



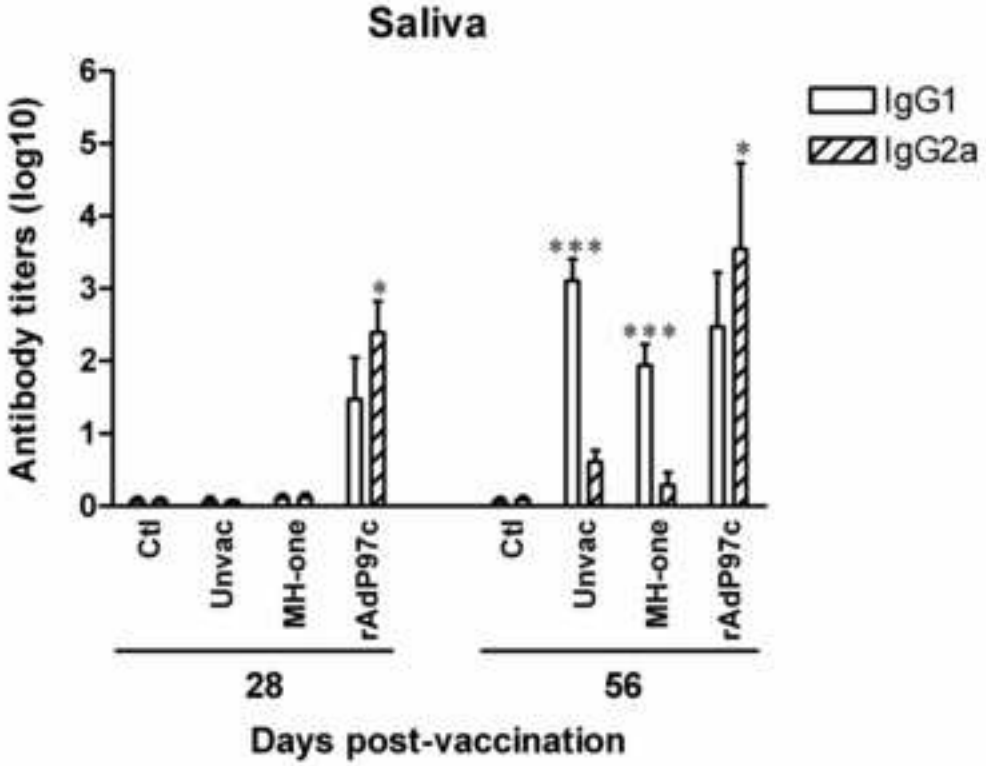


Figure 3

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Figure 3. P97c specific lymphocytes proliferation immune response.

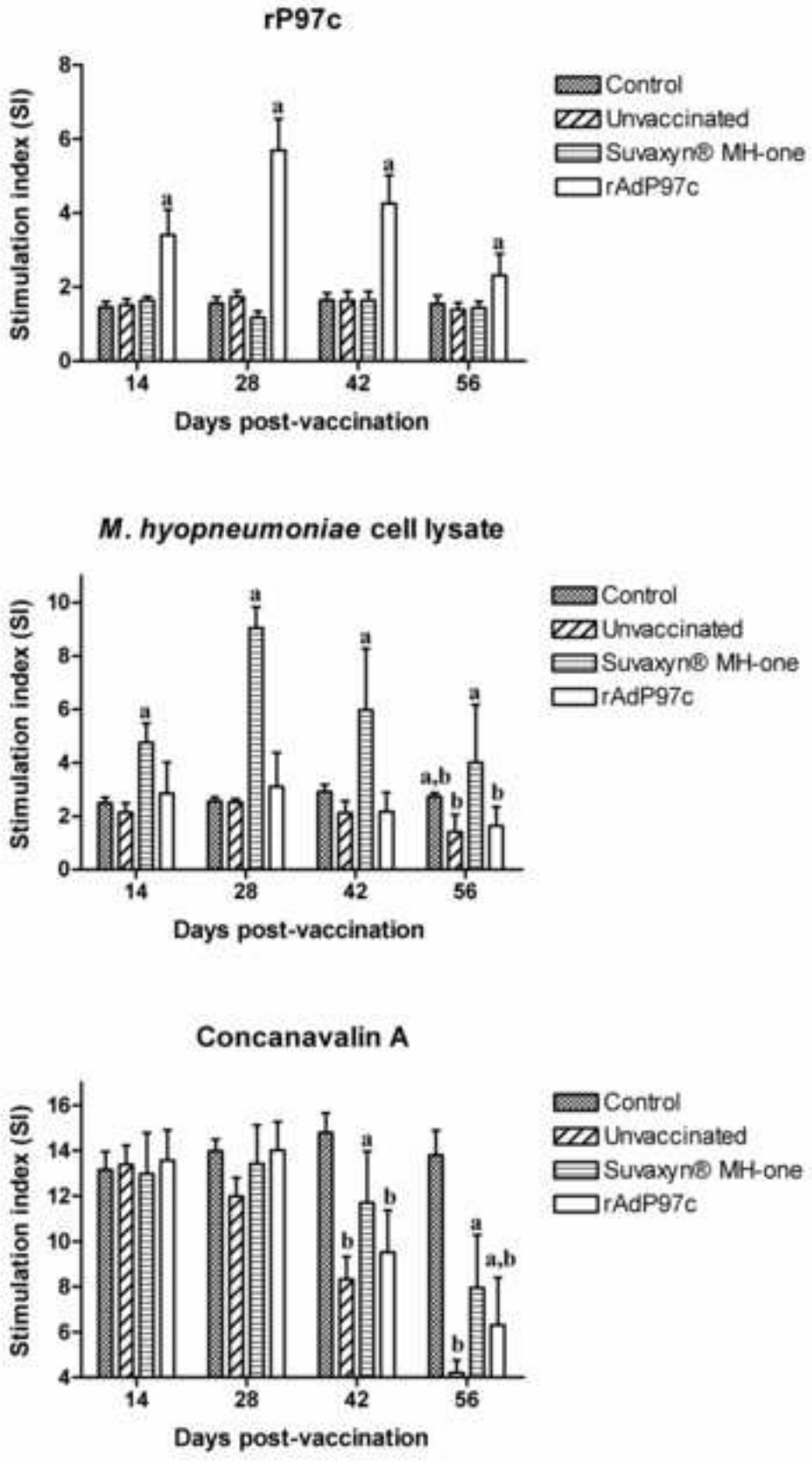

Figure 4
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Figure 4. Growth inhibition of *M. hyopneumoniae* by serum of vaccinated pigs.

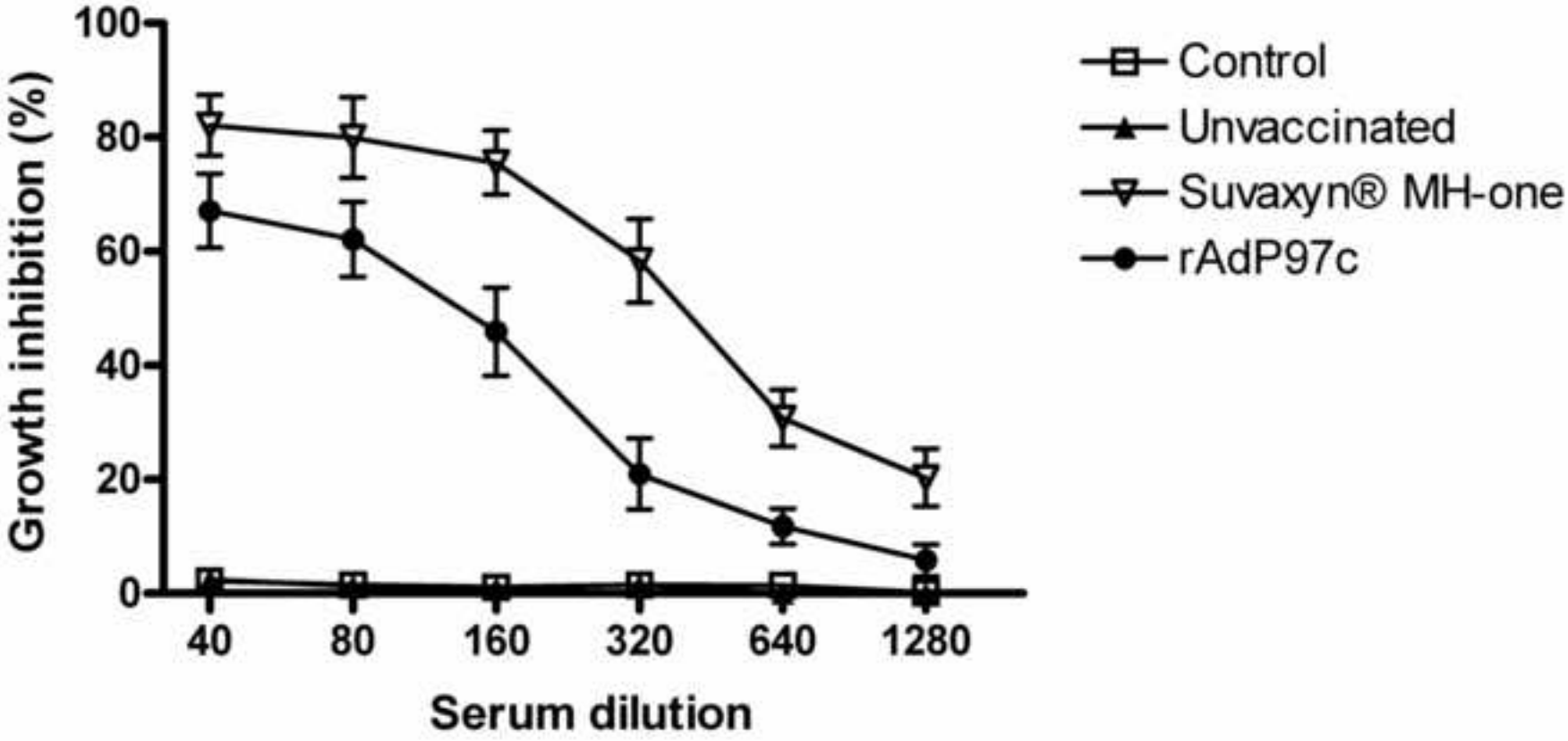

Figure 5
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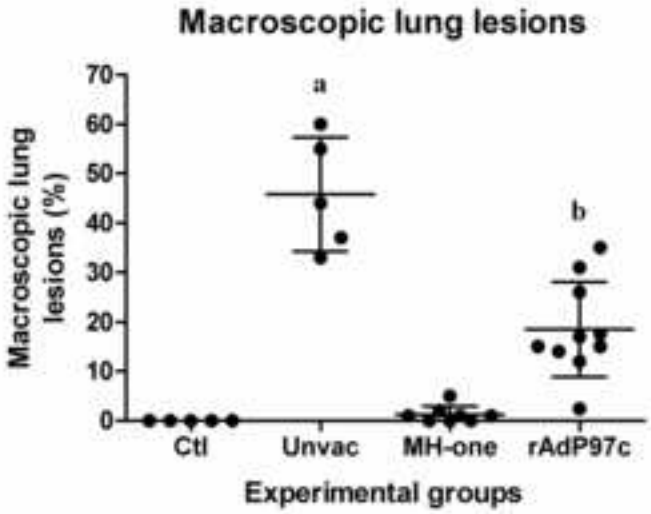
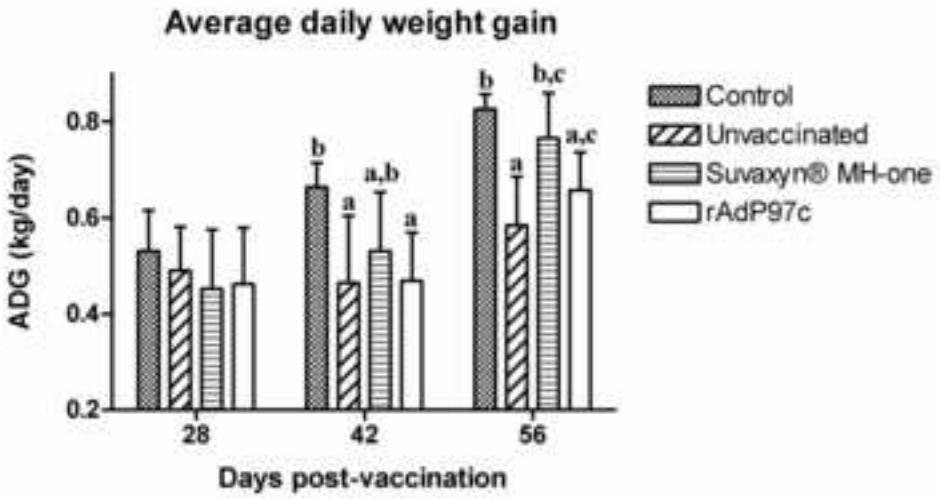
Figure 5. Level of protection induced in rAdP97c vaccinated pigs.





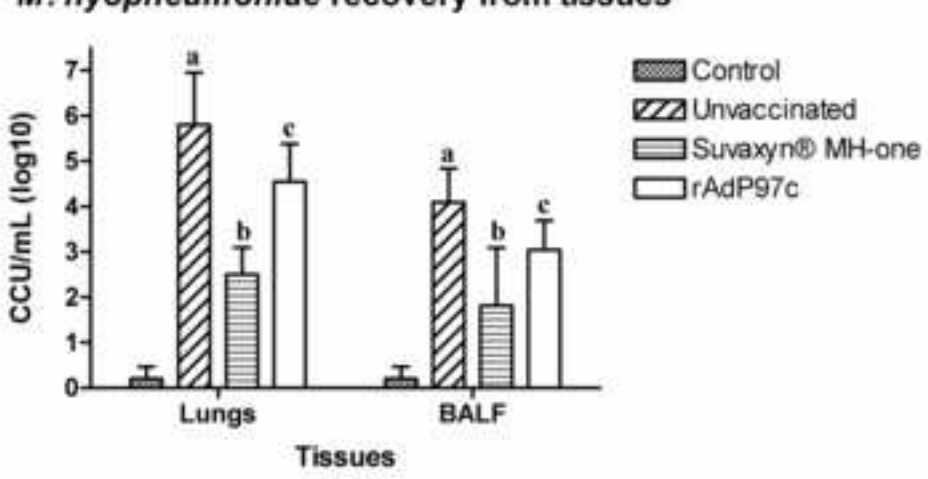


Figure 6
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Figure 6. Inflammatory immune responses in the bronchoalveolar lavage fluids.