# Vaccine

# Immunisation of mice with *Mycoplasma hyopneumoniae* antigens P37, P42, P46 and P95 delivered as recombinant subunit or DNA vaccines

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## ARTICLE INFO

## ABSTRACT

Porcine enzootic pneumonia (PEP), which is caused by the fastidious bacterium *Mycoplasma hyopneumoniae*, is one of the most economically important diseases in the pig industry worldwide. Commercial bacterins provide only partial protection; therefore, the development of more efficient vaccines against PEP is necessary. In this study, the cellular and humoral immune responses elicited by DNA and recombinant subunit vaccines based on the P37, P42, P46 and P95 antigens of *M. hyopneumoniae* were evaluated after the intramuscular inoculation of BALB/c mice. The expression of the cytokines INFγ, TNFα and IL1 was evaluated by real-time RT-PCR in splenocytes from vaccinated mice. All antigens delivered as subunit vaccines, especially P42 and P95, and the pcDNA3/P46 DNA vaccine were able to elicit strong immune responses. These vaccines induced cellular immune responses and the production of antibodies able to react with native *M. hyopneumoniae* proteins. Because both cellular and humoral immune responses were induced, P42 and P95 are promising candidates for a recombinant subunit vaccine and P46 is a promising candidate for a DNA vaccine against PEP.

## 1. Introduction

*Mycoplasma hyopneumoniae* is the causative agent of porcine enzootic pneumonia (PEP), a highly prevalent respiratory disease that causes major losses in pig farms worldwide. This disease is characterised by catarrhal pneumonia with chronic non-productive cough, low feed conversion and delays in weight gain, leading to high morbidity and low mortality [1]. In addition, pigs with PEP are predisposed to secondary infections [2].

PEP can be controlled with antibiotics and animal handling procedures. However, vaccination is considered the most efficient control measure [3]. Commercially available vaccines consist of inactivated whole-cell preparations and provide only partial protection [4,5]. Therefore, it is necessary to develop new alternative vaccines. Some studies have reported that DNA vaccines favour the Th1 immune response and thus promote a cellular immune response [6–8]. Despite the fact that the mechanism of *M. hyopneumoniae* pathogenesis has not been completely elucidated, it has been suggested that a cell-mediated immune response is important for disease control [5]. Thus, DNA vaccines represent a promising strategy for developing more effective vaccines against PEP.

After the sequencing and proteomic analysis of two *M. hyopneumoniae* strains [9], our research group produced and evaluated the immunogenicity and antigenicity of 35 secreted recombinant proteins [10–12] and six transmembrane recombinant proteins [13] expressed in *Escherichia coli*. Some of these proteins were identified as having the potential to be used as vaccine antigens, including the molecular chaperone DnaK (P42 heat shock protein) and the membrane surface protein P46. The latter has been tested as a DNA vaccine but only as part of a cocktail, not individually [6,14]. Two other recombinant proteins, P37-like ABC transporter substrate-binding lipoprotein and the outer membrane protein P95, which have not been evaluated previously, were also considered as potential antigens for use in subunit vaccines. None of these antigens have been evaluated as DNA vaccines.

In this work, the cellular and humoral immune responses elicited by the *M. hyopneumoniae* antigens P37, P42, P46 and P95, delivered as DNA or recombinant subunit vaccines, were evaluated in mice.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

*M. hyopneumoniae* strain 7448 was obtained from EMBRAPA (Concórdia-SC, Brazil). *E. coli* TOP10 was used as a host strain for cloning, and *E. coli* BL21(DE3)RIL was used as the expression strain.

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**Table 1**
*M. hyopneumoniae* CDS selected and the respective primers and restriction enzymes used for cloning and site-directed mutation.

| CDS[a] | Features | Primers | Restriction enzymes |
|---|---|---|---|
| MHP0360 For | P37 | CG<u>GGATCC</u>GATCAAAAAGACTGGGAAAAATT | *Bam*HI |
| MHP0360 For[b] | P37 | CGC<u>GGGATCC</u>AAAAAATGGACTGGGAAAAA | *Bam*HI |
| MHP0360 Rev | P37 | CGG<u>AATTC</u>TTAGTTAGATTCTGCATAAATCCG | *Eco*RI |
| MHP0360 Rev[b] | P37 | CGG<u>AATTC</u>TTAGTTAGATTCTGCATAAATCCG | *Eco*RI |
| MHP0360 FM | P37 | CATTTGCTTG**g**ACTGAAAGCAAA | |
| MHP0360 RM | P37 | GCTTTCAGT**c**CAAGCAAATGAA | |
| MHP0067 For | P42 | *CACC*ATGGCGCTTACAAGACTTAA | |
| MHP0067 For[b] | P42 | CGC<u>GGGATCC</u>CAACGATGGTTTCAGCAAAG | *Bam*HI |
| MHP0067 Rev | P42 | TGATTAATCCTGCTTGATTTCAGCAT | |
| MHP0067 Rev[b] | P42 | CCGG<u>AATTC</u>TTAATCCTGCTTGATTTCAGCA | *Eco*RI |
| MHP0513 For | P46 | *CACC*CTCACTCAGCAAG | |
| MHP0513 For[b] | P46 | CGG<u>GGTACC</u>TCACTATGGAGCAAGCTAAT | KpnI |
| MHP0513 Rev | P46 | TTAGGCATCAGGATTATCAACATTAGC | |
| MHP0513 Rev[b] | P46 | CGC<u>GGATCC</u>TTAGGCATCAGGATTATCAAC | *Bam*HI |
| MHP0513 FM | P46 | GTCCCAGGATG**g**AATTATGGAACT | |
| MHP0513 RM | P46 | ATAATT**c**CATCCTGGGACATAAACAG | |
| MHP0099 For | P95 | *CACC*AGCCGTCCTTTTTTTGAAA | |
| MHP0099 For[b] | P95 | CGC<u>GGGATCC</u>GAAGCATGGGTCCTTTTTTTG | *Bam*HI |
| MHP0099 Rev | P95 | TGATCAAAGTTTAAAAATCTCAGTTTCT | |
| MHP0099 Rev[b] | P95 | CCGGAATTCTCAAAGTTTAAAAATCTCAGTTTCTT | *Eco*RI |

For, forward primer; Rev, reverse primer; FM and RM, internal primers designed for site-directed mutagenesis. Mutated nucleotides are in bold and lowercase.

[a] NC_007332 GenBank® accession for *M. hyopneumoniae* strain 7448.

[b] Primers designed for cloning into pcDNA3.1 vector. The others were designed for cloning into pAE or PET200D TOPO. Italic nucleotides represent CACC sequence used for site-direct cloning into PET200D TOPO plasmid. Underlined nucleotides represent the restriction site of the enzyme.

The expression vectors Champion pET200D/TOPO and pcDNA3 were purchased from Invitrogen, and the expression vector pAE was obtained from Instituto Butantan [15].

## 2.2. Amplification of coding sequences and site-directed mutagenesis

The primers used were designed with VectorNTI 10 (Invitrogen) software based on the genomic sequence of *M. hyopneumoniae* strain 7448 (GenBank accession NC_007332) (Table 1). The Kozak sequence was added to the forward primers to generate DNA vaccines. The *M. hyopneumoniae* P37, P42, P46 and P95 coding sequences were amplified and purified according to the method of Simionatto et al. [11]. MHP0246 (P37) and MHP0513 (P46), which contained a TGA codon in their nucleotide sequences, were subjected to site-directed mutagenesis according to the method of Simionatto et al. [10], and the mutation was confirmed by DNA sequencing using the Dye Terminator DYEnamic ET Cycle Sequencing kit and a MegaBACE 1000 DNA sequencer (GE Healthcare).

## 2.3. Recombinant subunit vaccine development

Recombinant proteins were expressed in *E. coli* BL21(DE3)RIL, purified and quantified as described by Simionatto et al. [11]. Recombinant proteins were also characterised by Western blotting using an anti-histidine tag monoclonal antibody conjugated to peroxidase (Sigma Aldrich).

## 2.4. In vitro transfection of Vero cells

DNA vaccines (pcDNA3/P37, pcDNA3/P42, pcDNA3/P46, pcDNA3/P95 and empty vector pcDNA3) were purified using the NucleoBond® Xtra Maxi kit (Macherey Nagel), according to the manufacturer's instructions. These DNAs were used to transiently transfect Vero cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Immunofluorescence analysis using mouse polyclonal serum previously produced against recombinant proteins [12] and RT-PCR were performed to confirm the *in vitro* expression of the encoded antigens prior DNA vaccination.

## 2.5. Inoculation of mice with the vaccine formulations

Female BALB/c mice aged 6–8 weeks (8 animals per group) were immunised as follows: group 1, pcDNA3/P37; group 2, pcDNA3/P42; group 3, pcDNA3/P46; group 4, pcDNA3/P95; group 5, P37; group 6, P42; group 7, P46, group 8, P95; group 9, RespiSure® vaccine (Pfizer) (positive control); group 10, 100 µl of saline (negative control for subunit vaccines); and group 11, the pcDNA3 vector (negative control for DNA vaccines). The recombinant subunit vaccines and the saline were supplemented with 15% aluminium hydroxide adjuvant. Two doses of 50 µg of protein or DNA were administered intramuscularly (IM) separated by 21 days. To enhance the uptake of the plasmid DNA by muscle cells, sucrose (50 µl of 25% solution) was injected 30 min before inoculation with the DNA vaccines. Blood samples were collected from the retroorbital sinus at 0, 21, 42, 63 and 84 days after inoculation (DAI). After the last blood sample was collected, the spleens of these animals were collected to evaluate cytokine production. During the experiment, all animals were maintained in accordance with the recommendations of the Brazilian College of Animal Experimentation and the Ethics Committee for Animal Experimentation of the Federal University of Pelotas.

## 2.6. Assessment of the humoral immune response

The humoral immune responses generated by immunisation with recombinant proteins and DNA vaccines were assessed by Western blotting and ELISA using recombinant proteins or crude *M. hyopneumoniae* 7448 extract, as described by Simionatto et al. [12]. An IgG anti-mouse antibody horseradish peroxidase conjugate (Sigma Aldrich) and antibodies supplied with the IsoQuick™ kit (Sigma Aldrich) were used to evaluate the levels of IgG1 and IgG2a antibodies. Sera from mice were assessed individually, and all reactions were performed in triplicate. Sera from mice immunised with saline or the commercial vaccine were used as negative and positive controls, respectively.

## 2.7. Assessment of cytokine induction (INFγ, TNFα and IL1)

Total RNA from splenocyte cultures was extracted with the Trizol Reagent® according to the manufacturer's protocol. Then, cDNA
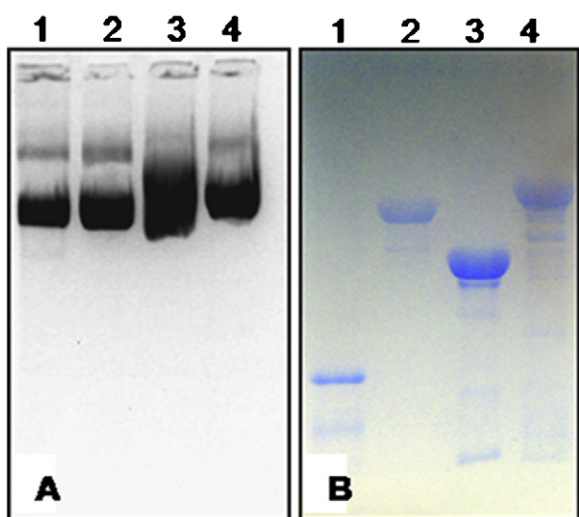
**Fig. 1.** (A) Electrophoresis in a 0.8% agarose gel of the four recombinant plasmids constructed for use as DNA vaccines. Lane 1, pcDNA3/P37; Lane 2, pcDNA3/P42; Lane 3, pcDNA3/P46; and Lane 4, pcDNA3/P95. (B) Electrophoresis in a 12% SDS-PAGE of the four recombinant proteins purified by affinity chromatography for use as subunit vaccines. Lane 1, P37 (20 kDa); Lane 2, P42 (42 kDa); Lane 3, P46 (35 kDa); and Lane 4, P95 (41 kDa).

was synthesised and subjected to real-time PCR. The PCR conditions used were as follows: a final volume of 20 $\mu$l containing 10 ng of cDNA, 3.5 mM $MgCl_2$, 3.1 pmol of primers, and 10 $\mu$l of Platinum Sybr green UDG (Invitrogen). The amplification was standardised in a 7500 Fast thermocycler (Applied Biosystems) using the following temperature program: 50 °C for 20 s, 95 °C for 10 min and 45 cycles of 15 s at 95 °C and 60 s at 60 °C. Melting curves were obtained using the following conditions: 15 s at 95 °C, 60 s at 60 °C, 30 s at 95 °C and 15 s at 60 °C. Primers to amplify cytokine genes were as previously described [16–18]. Primers were evaluated for efficiency, which was close to 100%, allowing their use in real-time PCR assays. Graphs of the relative expression were generated using the $2^{-\Delta\Delta Ct}$ method [19].

### 2.8. Statistical analysis

Significant differences in the ELISA results were identified by analysis of variance (ANOVA) using the Tukey test in SAS 9.1 [20]. A $P$ value of 0.05 or 0.01 was considered significant. Graphs were prepared in GraphPad Prism 5.

## 3. Results

### 3.1. Recombinant subunit and DNA vaccine development

All recombinant proteins (P37, P42, P46 and P95) were expressed in *E. coli* BL21(DE3)RIL and purified by affinity chromatography with sufficient purity and quantity for further studies (Fig. 1). The four selected targets were successfully amplified by PCR and cloned into pcDNA3. The obtained plasmids had high purities (A260 > 1.8 and <2.2) and yields ranging from 500 to 2500 $\mu$g/L of culture (Fig. 1). The presence of specific mRNA was verified by RT-PCR, and the expression of proteins was assessed by immunofluorescence analysis of Vero cells transfected with the recombinant vectors.

### 3.2. Immunogenicity of recombinant subunit and DNA vaccines

The total IgG antibody response induced by the recombinant vaccines was determined after intramuscular (IM) immunisation
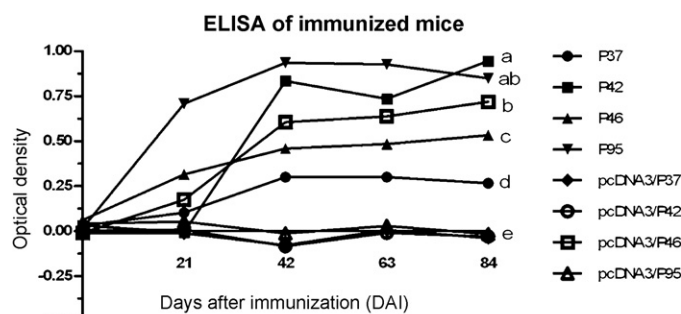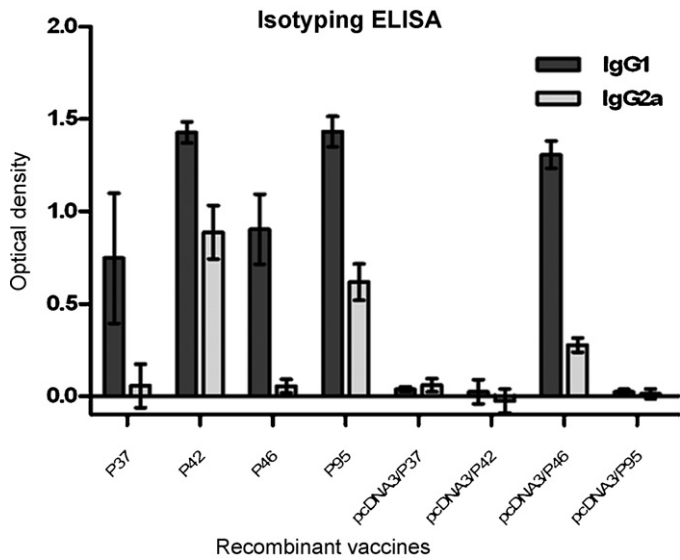


**Fig. 2.** Optical density (mean ± SD) obtained for the indirect ELISA using serum (diluted 1:50) from eight BALB/c mice immunised with the P37, P42, P46, P95, pcDNA3/P37, pcDNA3/P42, pcDNA3/P46 and pcDNA3/P95 vaccines. Serum was collected on days 0, 21, 42, 63 and 84 DAI. An anti-mouse IgG-peroxidase conjugate (diluted 1:4000) was used as the secondary antibody. The colorimetric reaction was started by adding o-phenylenediamine dihydrochloride (Sigma Aldrich) and hydrogen peroxide and was stopped with 2 M $H_2SO_4$. The optical densities (492 nm) for the subunit and DNA vaccines were subtracted from the optical density generated by the negative control (saline and pcDNA3, respectively). Small letters indicates the results of the ANOVA using the Tukey test, with $P < 0.01$ for day 84.

of BALB/c mice by indirect ELISA using recombinant proteins (Fig. 2). At 42 DAI, the sera of mice contained specific antibodies against the recombinant P37, P42, P46 and P95 proteins, with a $P$ value <0.01 for the Tukey test relative to preimmune serum. This response was constant until 84 DAI. The Serum of animals inoculated with saline (negative control) and the commercial vaccine (positive control) did not react with any recombinant proteins (data not shown). Among the recombinant subunit vaccines tested, the P42 and P95 vaccines elicited the strongest immune responses ($P < 0.01$) at 84 DAI. Among DNA vaccines, only the pcDNA3/P46 vaccine induced seroconversion. The level of seroconversion induced by the pcDNA3/P46 vaccine was statistically equal to that induced by the P95 recombinant subunit vaccine and was superior to that induced by the P46 recombinant subunit vaccine ($P < 0.01$). Although the P42 and P95 vaccines showed a mixed IgG1/IgG2a immune response, there was a predominance of the humoral immune response (IgG1) for all recombinant vaccines, even the pcDNA3/P46 vaccine (Fig. 3). The P42 vaccine elicited the highest IgG1 and IgG2a levels, followed by the P95 and pcDNA3/P46 vaccines.

The sera of mice immunised with the P42, P46, P95 and pcDNA3/P46 vaccines (84 DAI) and the commercial vaccine (positive control) significantly recognised the native proteins (Fig. 4) in the ELISA. In the Western blot analysis, the same result was observed for all sera except those from mice immunised with P46 vaccine, which was not reactive (data not shown).

### 3.3. Real-time PCR

Total RNA was extracted from the spleens of immunised mice and subjected to real-time PCR analysis. Both the recombinant subunit and DNA vaccines showed less or equal IL1 expression than the control (saline group and pcDNA3 group, respectively). On the other hand, the bacterin-vaccinated group (positive control) showed IL1 expression higher than that of the control (saline group) (Fig. 5A). The relative expression of TNF$\alpha$ was lower in the mice immunised with the bacterin and all recombinant vaccines except the pcDNA3/P42 and pcDNA3/P95 vaccines relative to the control (Fig. 5B). This negative effect on the IL1 and TNF$\alpha$ pro-inflammatory cytokine response was more pronounced in the groups vaccinated with P46, both as a recombinant subunit vaccine and a DNA vaccine. Furthermore, the DNA-vaccinated groups showed higher IL1 and TNF$\alpha$ expression than the groups that received the recombinant subunit vaccines.

**Fig. 3.** Optical density (mean ± SD) obtained by sandwich ELISA using individual sera (100 μg each well) from eight BALB/c mice immunised with the P37, P42, P46, P95, pcDNA3/P37, pcDNA3/P42, pcDNA3/P46 and pcDNA3/P95 vaccines. The sera were collected 84 days after inoculation. Anti-mouse IgG1 and IgG2a, supplied in the IsoQuick™ kit (Sigma Aldrich), were used as secondary antibodies. The colorimetric reaction was started by adding o-phenylenediamine dihydrochloride (Sigma Aldrich) and hydrogen peroxide and was stopped with 2 M $H_2SO_4$. The optical densities (492 nm) of the recombinant subunit and DNA vaccines were subtracted from the optical densities for the negative control (saline and pcDNA3, respectively) and the pre-immune sera (day 0).

All recombinant subunit vaccines and the pcDNA3/P46 DNA vaccine induced more INFγ expression than the control (Fig. 5C). INFγ is an important cytokine involved in cellular immune responses (Th1 type). As expected, pcDNA3/P46 elicited a stronger Th1 immune response than the recombinant proteins. Although the bacterin vaccine also induced INFγ expression, the expression level was statistically lower than that for all recombinant protein vaccines.
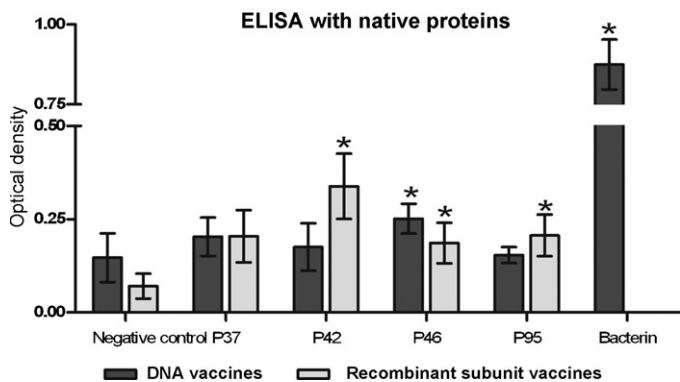


**Fig. 4.** Indirect ELISA analysis using native proteins from *M. hyopneumoniae* strain 7448 (1 μg each well) as the antigens and individual serum (diluted 1:20) from mice immunised with the recombinant vaccines (P37, P42, P46, P95, pcDNA3/P37, pcDNA3/P42, pcDNA3/P46, and pcDNA3/P95) collected 84 days after inoculation. An anti-mouse IgG-peroxidase conjugate (diluted 1:4000) was used as the secondary antibody. The colorimetric reaction was started by adding o-phenylenediamine dihydrochloride (Sigma Aldrich) and hydrogen peroxide and was stopped with 2 M $H_2SO_4$. The data show the optical density (492 nm) after subtraction from the value for the pre-immune serum (day 0). Treatments that differed from the negative control (saline for subunit vaccines and pcDNA3 for DNA vaccines) according to the Tukey test ($P < 0.01$) are marked with asterisks.
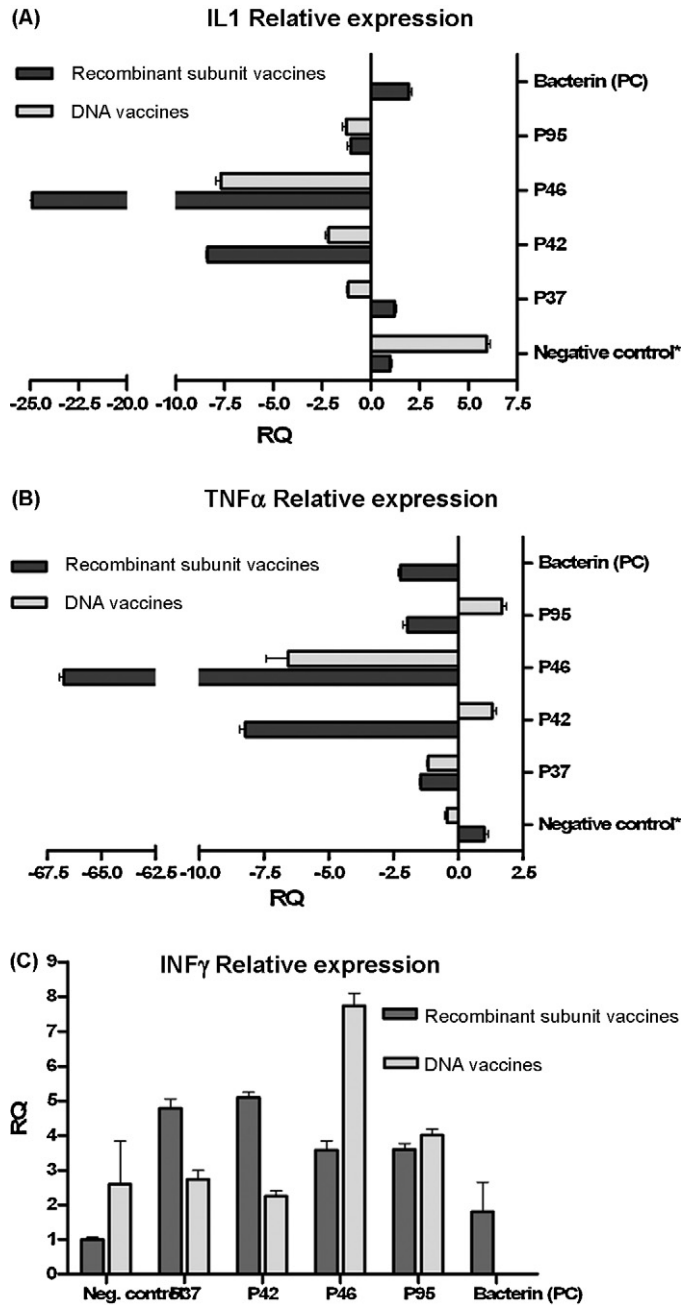


**Fig. 5.** Relative expression levels (mean RQ ± SD) of IL1 (A), TNFα (B) and INFγ (C). Spleens from BALB/c mice immunised with P37, P42, P46 and P95, delivered as recombinant subunit or DNA vaccines, were collected, and splenocytes were cultured and stimulated with recombinant P37, P42, P46 or P95 proteins, respectively. Total RNA from these cultures was used for real-time PCR analysis. The data show the expression levels relative to those for the negative control (*saline group for subunit vaccines and pcDNA3 vector for DNA vaccines). The values were normalised to the level of the GAPDH housekeeping gene.

## 4. Discussion

Although commercial vaccines are widely used worldwide, they provide only partial protection, highlighting the need for new vaccines with improved efficacy [4,5]. In this study, four immunogenic *M. hyopneumoniae* antigens, P37, P42, P46 and P95, were evaluated as subunit and DNA vaccine candidates. The analysis of the antibody responses in mice vaccinated with both vaccine formulations revealed that all subunit vaccines were able to induce specific IgG antibodies, but the pcDNA3/P46 vaccine was the only DNA vaccine

that induced a humoral immune response. This is the first report of the immune response induced by pcDNA3/P46 delivered alone.

It is not clear why the other DNA vaccine constructs failed to elicit serum IgG responses. The P37 protein was recognised by polyclonal antibodies in the immunofluorescence assay, and P95 mRNA was detected by RT-PCR in transfected Vero cells, indicating that these proteins were expressed. One possible explanation could be that the *in vivo* expression of these proteins was poor and that the quantities of antibodies induced by these proteins were not measurable. Furthermore, pcDNA3/P46 and the subunit vaccines for P42, P46 and P95 also induced antibodies able to recognise native proteins, as demonstrated in the ELISA (Fig. 4) and Western blot analysis. These results indicate that the folding of these proteins was most likely similar to that of the native proteins, with the appropriate epitopes exposed on the surface, unlike the proteins produced by pcDNA3/P37 and pcDNA3/P95. Thus, correct folding and/or other posttranslational modifications may have allowed pcDNA3/P46 to generate antibodies when other antigens delivered as DNA vaccines could not.

DNA vaccination has been demonstrated to induce both humoral and cellular immune responses [8]. The immune system modulation, the Th type and the IgG subclass distribution may be important for protection against enzootic pneumonia. Th1 cells can induce macrophages to destroy intracellular microorganisms more efficiently and also activate B cells to produce strongly opsonising antibodies such as IgG2a and IgG2b in mice. In contrast, Th2 cells induce B cells to proliferate and produce antibodies such as IgG1 and other types [21]. Therefore, we assayed the levels of the IgG1 and IgG2a subclasses and of INFγ produced by immunised mice with the recombinant vaccines to assess the type of Th induction of each vaccine. Protein vaccines are known to generally induce predominately Th2 responses with weak or no Th1 responses. Surprisingly, all subunit recombinant vaccines elicited INFγ levels higher than those induced by the negative control (Fig. 5C) and by all DNA vaccines except pcDNA3/P46. The P42 and P95 vaccines elicited higher IgG2a levels than the other vaccines and induced a mixed Th1/Th2-type immune response in mice, similar to what was observed when P42 was delivered as a DNA vaccine [6]. Thus, both vaccination strategies seem promising when using the P42 antigen. Heat shock proteins, such as P42, have been shown to be recognised by multiple B-cell and T-cell clones [6] and have also been used as adjuvants to enhance cell-mediated immune responses in DNA vaccines [22]. Thus, the nature of the protein could be responsible for the immune system modulation. However, it is important to note that the induction of Th1 and Th2 immune responses by a vaccine is species dependent and that the induction of antibodies in mice against *M. hyopneumoniae* is not correlated with protection. Therefore, further studies in pigs are necessary.

Interestingly, P46 use as a DNA vaccine generated more IgG1 and IgG2a antibodies and also induced more INFγ gene expression than when use as a subunit vaccine. It is important to note that the humoral immune response of mice vaccinated with pcDNA3/P46 was not higher ($P < 0.01$) but was more consistent among animals than the response to P46 delivered as a recombinant subunit vaccine. This result is in accordance with a recent study in which P46 elicited a serum IgG response in mice intramuscularly immunised with a DNA vaccine cocktail comprising the antigens P36, P46, NrdF, and P97 or P97R1 [14]. However, the authors of that previous study used the entire sequence of P46, whereas we omitted the first 300 nucleotides to avoid the need to mutate two additional TGA codons. It appears that the omitted sequence is not necessary to elicit immune responses.

The evaluation of local and systemic immune responses induced by the intramuscular injection of an *M. hyopneumoniae* bacterin into pigs showed that the TNF-α concentration in the BALF was higher in non-vaccinated challenged pigs than in vaccinated challenged pigs [5]. The presence of IL1 and TNFα in the BALF would thus appear to be associated with the development of pneumonic lesions in *M. hyopneumoniae*-infected pigs [23–25]. Therefore, reduction of the inflammatory reaction caused by *M. hyopneumoniae* is desirable. In the present study, all evaluated vaccines reduced or maintained the expression of IL1 and TNFα relative to the negative control; the P42 subunit vaccine and the P46 antigen delivered as a subunit or DNA vaccine were particularly effective (Fig. 5). We hypothesised that the reduction in expression of these cytokines was generated by IL-10, an immunosuppressive cytokine, thus suggesting specific activation of regulatory T cell. However, the reduction in the expression of these cytokines in non-challenged vaccinated mice does not necessarily indicate that the expression levels of these cytokines will also be reduced after the infection of vaccinated pigs with *M. hyopneumoniae*. Thus, this result should be confirmed in studies using vaccinated pigs that are challenged with virulent *M. hyopneumoniae*.

It may be possible that the type of immune response induced depends on the vaccine construct, the adjuvant used and the route of immunisation in addition to the nature of the antigen itself. Conceição et al. [26] reported that mice immunised with a recombinant subunit vaccine containing P97R1 fused to the B subunit of the heat-labile enterotoxin of *E. coli* induced Th1-biased immune responses when given intranasally but not when given intramuscularly (which induced Th2-biased responses). Chen et al. [7] showed that *Salmonella enteric* serotype Typhimurium *aroA*CS332 harbouring a eukaryotic or prokaryotic expression vector encoding P97R1 or NrdF induced antigen-specific Th1-biased immune responses in mice that were immunised orally. Mice immunised intranasally with a replicative-defective recombinant adenovirus expressing the C-terminal portion of *M. hyopneumoniae* P97 adhesin showed a mixed Th1/Th2-type response against P97 [27]. The mycobacterial ESAT-6 protein was able to enhance the mouse IFN-γ response to the *M. hyopneumoniae* P71 protein [28] and P71 DNA vaccines [29]. Therefore, it would be interesting to evaluate the differences in the Th1 and Th2 immune responses induced by pcDNA3/P46 when using other routes of vaccination.

It is also important to note that the commercial vaccine (Resp®Sure) induced antibodies in mice that were able to recognise native proteins of *M. hyopneumoniae* but did not induce antibodies able to recognise any of the recombinant proteins used in this study. In addition, this commercial vaccine did not induce INFγ gene expression. Similar results were reported by Marchioro et al. [13] and Simionatto et al. [12] using a wide range of recombinant proteins. The reason for the lack of recognition of the recombinant proteins by antibodies induced by the commercial vaccine is unknown. It is possible that the immunogenic proteins are present at low concentrations in this vaccine formulation. Although we have no confirmation that immune response to these proteins would induce any protection against infection, colonisation or disease in pigs after infection with *M. hyopneumoniae*, perhaps a stronger protective effect could be obtained by adding, to the commercial vaccine, immunogenic proteins such as those evaluated in this study.

In summary, we demonstrated that intramuscular immunisation with the pcDNA3/P46 vaccine delivered alone was able to induce both Th1- and Th2-biased immune responses, with an increase in the INFγ level but decreases in the levels of IL1 and TNFα, which are responsible for the lung lesions that occur during *M. hyopneumoniae* infection. Additionally, P42 and P95, antigens that had not been previously evaluated regarding the induction of cellular immune responses, elicited high IgG2a and INFγ levels when delivered as subunit vaccines. However, it is well known that the immune responses of mice may not always be extrapolated to other species. In addition, immune responses are not only dependent on the animal species but may also depend on the mouse strain

used. Therefore, further studies in pigs are required to evaluate the ability of these vaccines to control enzootic pneumonia.

## Acknowledgements

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