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Immune responses elicited by *Mycoplasma hyopneumoniae* recombinant antigens and DNA constructs with potential for use in vaccination against porcine enzootic pneumonia

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ABSTRACT

Mycoplasma hyopneumoniae is the etiological agent of porcine enzootic pneumonia (PEP) and causes major economic losses to the pig industry worldwide. Commercially available vaccines provide only partial protection and are relatively expensive. In this study, we assessed the humoral and cellular immune responses to three recombinant antigens of *M. hyopneumoniae.* Immune responses to selected domains of the P46, HSP70 and MnuA antigens ($P46_{102–253}$, $HSP70_{212–601}$ and $MnuA_{182–378}$), delivered as recombinant subunit or DNA vaccines, were evaluated in BALB/c mice. All purified recombinant antigens and two DNA vaccines, pcDNA3.1(+)/$HSP70_{212–601}$ and pcDNA3.1(+)/$MnuA_{182–378}$, elicited a strong humoral immune response, indicated by high IgG levels in the serum. The cellular immune response was assessed by detection of IFN-γ, IL-10 and IL-4 in splenocyte culture supernatants. The recombinant subunit and DNA vaccines induced Th1-polarized immune responses, as evidenced by increased levels of IFN-γ. All recombinant subunit vaccines and the pcDNA3.1(+)/$MnuA_{182–378}$ vaccine also induced the secretion of IL-10, a Th2-type cytokine, in large quantities. The mixed Th1/Th2-type response may elicit an effective immune response against *M. hyopneumoniae*, suggesting that $P46_{102–253}$, $HSP70_{212–601}$ and $MnuA_{182–378}$ are potential novel and promising targets for the development of vaccines against PEP.

1. Introduction

Mycoplasma hyopneumoniae is the etiological agent of porcine enzootic pneumonia (PEP), a respiratory disease characterized by chronic coughing, poor growth rate and decreased feed conversion efficiency [1]. In addition, *M. hyopneumoniae* facilitates the respiratory tract invasion by other pathogens, causing significant economic losses to the pig industry [2].

Experimental PEP vaccine protocols involving inactivated whole cells (bacterins) [3–6] have been unable to prevent transmission or the establishment of the bacteria in the lungs [7–9]. Moreover, *in vitro* growth of *M. hyopneumoniae* for bacterin preparation requires a rich culture medium and is a time consuming process [10], increasing vaccine final cost.

Given the limitations of bacterins, which also include a lack of information regarding the antigens expressed in different preparations, the immunological characterization of novel *M. hyopneumoniae* antigens is still necessary. Recombinant formulations including individual *M. hyopneumoniae* antigens and multivalent vaccines have been shown to elicit both humoral and cellular immune responses in mice [11–13]. A smaller number of recombinant vaccine protocols have been tried so far in pigs, with limited success [14–16].

Another promising strategy for PEP vaccine development involves the use of DNA plasmids encoding potential antigens. Immunization with DNA constructs has been shown to induce protective cellular and humoral immune responses [17]. Moreover,

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Table 1
Primers used for DNA amplification and cloning steps, nucleotide length of the cloned CDS segments and amplicon length for each recombinant antigen.

Selected CDS	Specific sequence (5′–3′)	CDS length (bp)	Amplicon length (bp)
$P46_{102–253}$	F: *TGGTTCCGCGTGGATCCCCG*CTCACTCAGCAAGCTAATTT[a]	456	500
	R: *GCGAGGCAGATCGTCAGTCAGTCA*TCCTGGGACATAAACAGC[a]		
	F: *ATCCACTAGTCCAGTGTGGTGGAATGCCATGGTT*CTCACTCAGCAAGCTAATTT[b]		519
	R: *CAACAGATGGCTGGCAACTAGAAGGTTA*TCCTGGGACATAAACAGC[b]		
$HSP70_{212–601}$	F: *TGGTTCCGCGTGGATCCCCG*CTTGTTAAAAAAATCAAAGAAGA[a]	1167	1211
	R: *GCGAGGCAGATCGTCAGTCAGTCA*CAGCATCAATTGTGTTTG[a]		
	F: *ATCCACTAGTCCAGTGTGGTGGAATGCCATGGTT*CTTGTTAAAAAAATCAAAGAAGA[b]		1230
	R: *CAACAGATGGCTGGCAACTAGAAGGTTA*CAGCATCAATTGTGTTTG[b]		
$MnuA_{182–378}$	F: *CCGCGTGGATCTGAATTCCCGGGTC*CTTGCAGCCGTTAAATTT[c]	586	635
	R: *GCGAGGCAGATCGTCAGTCAGTC*AATTCTGACTGTTTTGGCC[c]		
	F: *ATCCACTAGTCCAGTGTGGTGGAATGCCATGGTT*CTTGCAGCCGTTAAATTT[b]		649
	R: *CAACAGATGGCTGGCAACTAGAAGGTTA*ATTCTGACTGTTTTGGCC[b]		

Primers: F, forward; R, reverse. Italic letters correspond to plasmid homologous sequences: [a] pGEX4T3; [b] pcDNA3.1(+); and [c] pGEX4T1. The Kozak sequences were added to the 5′ end of the specific sequences of the forward primers (underlined) for to cloning into pcDNA3.1(+) vector.

DNA constructs are stable, easy to handle and can be administered by various routes [11]. DNA vaccines encoding *M. hyopneumoniae* antigens [11,13,18] have been studied in mice.

Previous studies on recombinant proteins [19,20], the sequencing and analysis of *M. hyopneumoniae* genomes [21–24], and proteomic surveys [25,26] have allowed the identification of several genes encoding antigenic proteins and/or involved in pathogenicity. For instance, P46, a surface protein, and the heat shock protein HSP70 have previously been identified as *M. hyopneumoniae* antigens, while the MnuA nuclease was predicted as a surface virulence factor. HSP70, despite its conservation, has been proposed as a vaccine antigen for different pathogens, and, in some cases its use has conferred protection against infection and/or controled bacterial growth [27,28].

In this work, we selected domains of the *M. hyopneumoniae* P46, HSP70 and MnuA proteins and assessed their potential as targets for use in vaccination against PEP. These domains were selected based on their predicted extracellular localization, relative hydrophilicity and antigenic potential. Immune responses to the P46, HSP70 and MnuA domains delivered as recombinant subunit or DNA vaccines were evaluated in mice. Our results demonstrated that the three recombinant subunit vaccines and two of the DNA vaccines (based on HSP70 and MnuA coding sequences) induced strong humoral immune responses and that all six vaccine constructs elicited different levels of cellular immune responses, which were predominantly of the Th1-type.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli BL21 pLysE (Novagen) [29] and BL21 Star (Invitrogen) [30] strains were used to express the recombinant antigens. The *E. coli* KC8 strain was used for all of the cloning steps [31].

2.2. DNA constructs

M. hyopneumoniae strain 7448 was obtained from EMBRAPA Suínos e Aves (Concórdia, Brazil). The coding DNA sequences (CDS) of the P46 (MHP7448_0513), HSP70 (MHP7448_0067) and MnuA (MHP7448_0580), available from the NCBI gene database (http://www.ncbi.nlm.nih.gov), were analyzed with TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), Phobius (http://www.ebi.ac.uk/Tools/pfa/phobius/), TopPred 1.10 (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred) and Predicting Antigenic Peptides (http://imed.med.ucm.es/Tools/antigenic.pl) softwares to predict extracellular domains, hydropathicity, and antigenicity. *M. hyopneumoniae* TGA codons were avoided in order to make the heterologous expression more straightforward.

The CDS segments selected encode amino acid residues 102–253 of P46 ($P46_{102–253}$), 212–601of HSP70 ($HSP70_{212–601}$) and 182–378 of MnuA ($MnuA_{182–378}$) and were amplified using genomic *M. hyopneumoniae* 7448 DNA, with the primers described in Table 1. The amplicons were cloned by *in vivo* homologous recombination [31] into pGEX-4T1 or pGEX-4T3 prokaryotic expression vectors (GE Healthcare), linearized by *Xho*I and *Eco*RI restriction enzymes, respectively, and into the eukaryotic expression vector pcDNA3.1(+) (Invitrogen), linearized by *Xho*I and *Eco*RV restriction enzymes.

All recombinant plasmids were sequenced using the Dyenamic ET Dye Terminator Cycle Sequencing kit for MegaBace DNA Analysis Systems (GE Healthcare). For DNA vaccines, the recombinant plasmids, namely pcDNA3.1(+)/$P46_{102–253}$, pcDNA3.1(+)/$HSP70_{212–601}$ and pcDNA3.1(+)/$MnuA_{182–378}$ were purified using an Endofree Plasmid Mega kit (Qiagen) according to the manufacturer's instructions and quantified by Qubit™ fluorometer (Invitrogen).

2.3. Expression and purification of recombinant antigens

The $P46_{102–253}$, $HSP70_{212–601}$ and $MnuA_{182–378}$ polypeptides were expressed as fusion proteins with glutathione *S*-transferase (GST) and purified by affinity chromatography followed by thrombin cleavage [32].

The $P46_{102–253}$ and $MnuA_{182–378}$ fusion proteins were solubilized with sarkosyl detergent. The $MnuA_{182–378}$ fusion protein demonstrated inefficient cleavage with thrombin. Therefore, all experiments involving $MnuA_{182–378}$ were performed using the complete fusion protein $MnuA_{182–378}$-GST and included a negative control performed with purified GST. The antigens were quantified by Qubit™ fluorometer.

2.4. Expression of vaccine DNA constructs in cultured mammalian cells

Transient transfection of COS-7 cells with 10 μg of each purified DNA vaccine construct was performed using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. *In vitro* expression of the recombinant antigens prior to DNA immunization was analyzed by immunoblot with polyclonal sera (see Section 2.5).

2.5. Immunization of mice with recombinant subunit and DNA vaccines

Twelve-week-old female BALB/c mice (5 mice/group) were immunized with purified recombinant subunit and DNA vaccines.