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Immune responses following vaccination of pigs and mice against *Mycoplasma hyopneumoniae*

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List of abbreviations

ANOVA	Analysis of variance
BAL	Bronchoalveolar lavage
BLN	Bronchial lymph nodes
BSA	Bovine serum albumin
CT	Cholera toxin
DAB	3,3' Diaminobenzidine tetrahydrochloride
DPI	Days post inoculation
EDTA	Ethylenediaminetetraacetic acid
EP	Enzootic pneumonia
ELISA	Enzyme-linked immunosorbent assay
ELIspot	Enzyme-linked immunosorbent spot
GM1	Monosialotetrahexosylganglioside
H ₂ SO ₄	Sulphuric acid
HRP	Horseradish peroxidase
ICE	Integrative conjugal element
IF	Immunofluorescence
Ig	Immunoglobulin
IFN- γ	Interferon gamma
IL	Interleukin
IM	Intramuscular
IN	Intranasal
IPTG	Isopropyl β -D-1-thiogalactopyranoside
kDa	kiloDalton
LB	Luria-Bertani
LTB	Subunit B of heat-labile enterotoxin of <i>Escherichia coli</i>
MHC	Major histocompatibility complex
NrdF	Ribunocleotide reductase

OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PI	Pre inoculation
PMSF	Phenylmethanesulfonylfluoride
PRDC	Porcine respiratory disease complex
PRSSV	Porcine reproductive and respiratory syndrome virus
qPCR	Quantitative PCR
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
SEM	Standard error of means
S-IgA	Secretory IgA
SPF	Specific Pathogen Free
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TGF	Transforming growth factor
Th	T helper cells
TLR	Toll-Like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor

CHAPTER 1.

GENERAL INTRODUCTION

1.1 Review of the Literature

Respiratory diseases cause major problems for pig herds all over the world. Enzootic pneumonia (EP) is one of the most important respiratory diseases in pigs. It is highly prevalent and causes considerable economic losses for both pig producers and the entire industry. The losses are mainly due to decreased performance of the pigs and increased antimicrobial use (Maes et al., 2008).

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary etiological agent of EP (Maes et al., 1999), and it is one of the main pathogens involved in the porcine respiratory disease complex (PRDC) (Thacker and Minion, 2010). Apart from infections with *M. hyopneumoniae*, PRDC results from infections with other bacterial (*Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, *Pasteurella multocida*, *Streptococcus suis* and *Bordetella bronchiseptica*) and viral (porcine reproductive and respiratory syndrome virus – PRRSV, porcine circovirus type 2, Aujeszky's disease virus, swine influenza virus – SIV and porcine respiratory coronavirus) pathogens. PRDC is clinically characterized by coughing, retarded growth rates, inefficient feed conversion, anorexia, fever and dyspnea (Thacker and Minion, 2010). Although animals of all ages are susceptible to *M. hyopneumoniae* infection, animals in the growing and finishing phase are mostly affected. However, in herds without immunity, clinical disease following *M. hyopneumoniae* infections can affect pigs from all age groups, including suckling pigs and breeding animals (Sibila et al., 2007).

Several methodologies are used to diagnose infections caused by *M. hyopneumoniae*. Clinical signs and pathological lesions are used for a presumptive diagnosis of EP, but further laboratory tests are required for a conclusive diagnosis. Although cultivation of *M. hyopneumoniae* is considered the gold standard for diagnosis, this method is not routinely used (Sibila et al., 2009). This is mainly because isolation of the pathogen in Friis medium is very difficult (Friis, 1975). In addition, the presence of other mycoplasma species may decrease the growth of *M. hyopneumoniae*, rendering their isolation even more difficult. Thus, failure to cultivate this organism from potentially infected animals cannot rule out the infection in the herd (Thacker, 2004). Immunofluorescence (IF) testing (Kobisch et al., 1978) is also frequently performed. Serologic tests are commonly used to monitor the health status of herds, but serology is not suited for diagnosis at the individual animal level (Sorensen et al., 1997). Currently, PCR testing is the most sensitive technique for detecting infections caused by *M. hyopneumoniae* (Calsamiglia et al., 1999).

The control of EP should focus on the optimization of management practices and housing conditions (Maes et al., 2008), the use of antimicrobial medication (Vicca et al., 2004) and vaccination. Several commercial vaccines, consisting of the inactivated adjuvanted whole cells of *M. hyopneumoniae* are available and worldwide used. These vaccines have been proven to be effective in reducing the clinical signs, but only partial protection against the development of lesions is obtained (Haesebrouck et al., 2004).

In this review, the characteristics of *M. hyopneumoniae* related to pathogenesis, immune response, lesions and control measures will be discussed. Furthermore, special emphasis will be placed on vaccination strategies, including the use of reverse vaccinology approaches.

1.1.1 Aetiology

Mycoplasmas belong to the Mollicutes class and differ phenotypically from other bacteria by their diminutive size and absence of cell wall (Razin et al., 1998). Despite fundamental differences between mycoplasmas and other bacteria, many aspects of mycoplasmal molecular biology are similar to those of gram-positive bacteria (Thacker and Minion, 2010). Mycoplasmas are the smallest free-living self-replicating microorganisms. They also have small genomes (580 -1350 kb) with a high A + T content (about 70%). The whole genome of four *M. hyopneumoniae* strains (J, 232, 7448 and 168) has been sequenced (Liu et al., 2011; Minion et al., 2004; Vasconcelos et al., 2005).

Because of their small genome, mycoplasmas undergo limited metabolism and have few biosynthetic pathways (Dybvig and Voelker, 1996). The lack of biosynthetic pathways implies that they need to obtain amino acids, purines and pyrimidines, and membrane components from their growth environment (Thacker and Minion, 2010). In addition, *in vitro* cultivation is quite fastidious, and most species have never been cultivated (Razin et al., 1998). *M. hyopneumoniae* can be cultivated *in vitro*, but it requires serum enriched media such as Friis medium (Friis, 1975). The cultivation and isolation of *M. hyopneumoniae* are laborious, time-consuming and often not successful, so isolation is not used for routine diagnosis. Moreover, there is frequent contamination with other mycoplasmas such as *M. hyorhinis* (present in the respiratory tract of pigs) and *M. flocculare* (a non-pathogenic species that has similarities with respect to morphology, growth and antigenicity of *M. hyopneumoniae*) (Thacker and Minion, 2010).

The fastidious nature of mycoplasmas and the lack of genetic systems to elucidate protein structures and functions have hampered understanding of their biology. In addition, studies suggest that mycoplasmas have complex systems of recombination that are able to create a high rate of antigenic diversity (Dybvig and Voelker, 1996). Indeed, *Mycoplasma* species have developed strategies to vary their surface topography to avoid detection and eradication by the host immune response (Browning et al., 2011). A previous study has indicated a complex transcriptional organization of the *M. hyopneumoniae* genome and suggested the likely existence of yet unknown mechanisms involved in transcriptional regulation (Siqueira et al., 2011). *M. hyopneumoniae* can produce adhesins, modulins, aggresins and impedins that allow adhesion and modulation of the host immune system (Asai et al., 1996; Henderson et al., 1996; Muneta et al., 2008). Besides this, studies revealed that surface antigens of *M. hyopneumoniae* are proteolytically processed upon translocation across

the membrane (Bogema et al., 2011; Burnett et al., 2006; Djordjevic et al., 2004; Wilton et al., 2009). The ability of *M. hyopneumoniae* to selectively cleave its secreted proteins provides this pathogen with a remarkable capacity to alter its surface architecture.

Molecular techniques demonstrated that *M. hyopneumoniae* shows a large diversity at genomic (Stakenborg et al., 2006; Strait et al., 2008) and proteomic level (Calus et al., 2007). Vicca et al. (2003) showed that there are also differences in virulence. These authors classified isolates as low, moderately and highly virulent, based on the outcome of experimental infections. Villarreal et al. (2011a) demonstrated that infection with low virulent *M. hyopneumoniae* isolates did not prevent infection and disease against a subsequent infection with a highly virulent isolate. However, further research is necessary to investigate the clinical importance of these differences between isolates.

1.1.2 Pathogenesis

M. hyopneumoniae is a host specific pathogen that only infects pigs. The pathogenesis and especially possible virulence factors of *M. hyopneumoniae* are not yet fully known.

M. hyopneumoniae may infect animals via the inhalation of muco-respiratory droplets expelled during bouts of coughing from *M. hyopneumoniae*-infected animals (Desrosiers, 2001). Upon inhalation, *M. hyopneumoniae* must overcome the mucociliary escalator and transverse layers of heavily glycosylated mucins. These are produced as decoys for bacterial adhesins that exploit surface glycoconjugates and extracellular matrix components during colonization. *M. hyopneumoniae* adheres to the ciliated respiratory epithelia where it initially causes ciliostasis, destruction of cilia and possibly epithelial cell death (Debey and Ross, 1994).

The ability to adhere tightly to respiratory cilia is a key strategy to overcome the mucociliary host defense (Figure 1) (Tajima and Yagihashi, 1982; Zhang et al., 1994).

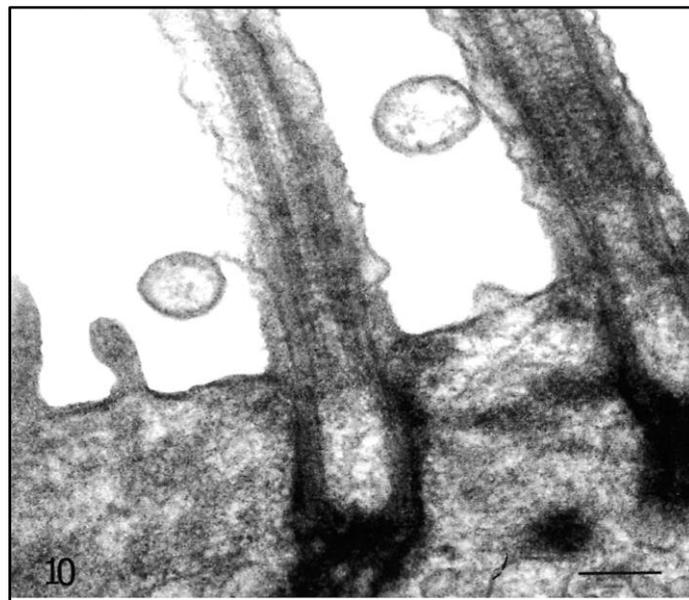


Figure 1: Transmission electron microscopy images of lung tissue from a pig infected with *M. hyopneumoniae*. Mycoplasma organisms are seen in close association with cilia (Sarradell et al., 2003).

Adhesion of the organism to tracheal, bronchial and bronchiolar epithelium, stimulation of a prolonged inflammatory reaction, suppression and modulation of the innate and adaptive immune responses are recognized as important steps in the pathogenesis of

infections with this organism (Thacker and Minion, 2010). As a result, infected animals are more susceptible to infections with other respiratory pathogens (Sorensen et al., 1997).

The exact method of adherence of the organism to the cilia has not been fully elucidated. A number of proteins involved in adherence have been identified. *M. hyopneumoniae* multifunctional adhesins belonging to the P97/P102 families include Mhp182 (P102), Mhp183 (P97), Mhp 493 (P159), Mhp 494 (P216), Mhp 683 (P135), Mhp 271, Mhp 107 and Mhp 108 (P116). These adhesins are expressed during broth culture (Burnett et al., 2006; Djordjevic et al., 2004; Pinto et al., 2007a; Wilton et al., 2009; Zhang et al., 1995) and *in vivo* in experimentally infected pigs (Adams et al., 2005). Fragments of P97 (Zhang et al., 1995), P216 (Wilton et al., 2009), P102 (Seymour et al., 2012), Mhp271 (Deutscher et al., 2012), Mhp107 (Seymour et al., 2011) and Mhp683 (Bogema et al., 2011) have been shown to bind porcine cilia.

P97 is the first identified adhesin of *M. hyopneumoniae*. It recognizes receptors present on the cilia of respiratory epithelial cells and is considered a virulence factor (Zhang et al., 1995). The C-terminal portion of P97, identified as R1, is responsible for cilium binding. Eight R1 repeating units are required for this binding. Variation due to the addition or subtraction of repeated amino acids may result in alteration of the protein, which may interfere with the recognition by the immune system (Minion et al., 2000). However, other factors or additional proteins are also required for adherence (Hsu and Minion, 1998).

The P102 protein is part of the same operon as P97 and its expression also appears to be associated with binding of the pathogen to cilia. Studies also suggest its participation in virulence since it is expressed *in vivo* during infection (Adams et al., 2005). Apart from these findings, the role of P102 and its paralogs has not been previously elucidated. Seymour et al. (2010) examined the surface expression and proteolytic processing of the P102 paralog P116, an extensively processed protein in *M. hyopneumoniae* strain 232. The results suggest that this protein is a multifunctional virulence determinant involved in the *M. hyopneumoniae* disease process.

Indeed, P97/P102 paralog family members on the cell surface of *M. hyopneumoniae* are targets of endoproteolytic processing events that generate a substantial combinatorial repertoire of cleavage fragments (Bogema et al., 2011; Deutscher et al., 2012; Wilton et al., 2009), suggesting that these endoproteolytic fragments might influence how *M. hyopneumoniae* interacts with its host. Endoproteolytic processing is a well-known mechanism used by bacterial pathogens to convert immature pre-proteins into mature functioning molecules. However, in the *M. hyopneumoniae* P97/P102 adhesin families,

posttranslational cleavage is extensive and unorthodox. Most of the P97/P102 family members previously examined undergo at least one cleavage event and in some cases two or more cleavage events (Bogema et al., 2011; Burnett et al., 2006; Djordjevic et al., 2004; Pinto et al., 2007a; Pinto et al., 2009; Wilton et al., 2009). Recently, a protease has been identified in *M. hyopneumoniae* which is responsible for processing the P97 and P102 family of cilium adhesins. It recognizes a peptide motif with a sequence similar to TTKF↓QE. This finding has facilitated the prediction of cleavage sites among members of these families (Bogema et al., 2011). However, the biological rationale for cleavage and the protease (s) responsible are not yet determined. Endoproteolytic processing significantly increases surface protein diversity by altering the presentation of functional domains on the cell surface and may be a mechanism used to regulate the adhesion to host tissues, potentiate host cell invasion, and avoid immune clearance (Bogema et al., 2012).

P102 and P97 are proteins with multifunctional binding abilities, which are also able to recruit plasminogen and fibronectin to the surface of *M. hyopneumoniae*. Binding of plasminogen and fibronectin to the surface of *M. hyopneumoniae* is dose dependent and saturable (Deutscher et al., 2012; Seymour et al., 2011; Seymour et al., 2012). Plasminogen is freely available in the bronchoalveolar fluid of the porcine lung, and *M. hyopneumoniae* displays surface receptors that bind plasminogen in a process that facilitates conversion to plasmin by mammalian plasminogen activators (Seymour et al., 2012). Consequently, *M. hyopneumoniae* colonizing the ciliated epithelia is likely to sequester plasminogen on its cell surface and facilitates its conversion to plasmin. This process is likely to have ramifications for tissue invasion and systemic infection (Bogema et al., 2012).

Although *M. hyopneumoniae* is primarily a respiratory pathogen, it has also been isolated from internal organs, suggesting that spread may occur via the lymphatic or blood circulation (Marois et al., 2007). The agent was re-isolated from the liver, spleen and kidneys of experimentally infected and contact pigs (Le Carrou et al., 2006; Marois et al., 2007) and *M. hyopneumoniae* DNA was detected in these same tissues (Woolley et al., 2012). However, this spread within the body appears to be transient and is likely not involved in the development of EP (Marois et al., 2007; Woolley et al., 2012).

It has been shown that *M. hyopneumoniae* has a modulating effect on the immune system, although the nature of this modulation is not well understood (Simecka, 2005). Alveolar macrophages and lymphocytes, stimulated by *M. hyopneumoniae*, produce pro-inflammatory cytokines that play a role in lung lesion development and lymphoid hyperplasia (Rodriguez et al., 2004). In general, mycoplasmas are capable of evading the host's natural

defenses. It is known that some pathogenic species use their genetic machinery to alter surface antigens, thus diverting the host immune response and allowing for chronic infection (Razin et al., 1998).

Data generated by sequencing and comparative analysis of three strains of *M. hyopneumoniae* allowed the identification of strain-specific regions that may be related to pathogenicity (Minion et al., 2004; Vasconcelos et al., 2005). The presence of an integrative conjugal element (ICE) was revealed in two strains that were pathogenic (7448 and 232) and is absent in the non-pathogenic J strain. This result suggests that the ICE, as a mobile DNA element, is probably involved in genetic recombination events, which can be related to the host adaptation and pathogenicity of the strain. Nevertheless, the transference of ICE between cells remains to be demonstrated experimentally (Pinto et al., 2007b).

1.1.3 Immune response

Immune responses in mycoplasma disease may be involved in pathogenesis. Although immunity is known to control and prevent infections, immune responses can also promote the inflammatory lesions associated with mycoplasma disease (Razin et al., 1998). The exact immune responses during infection of pigs with *M. hyopneumoniae* are not fully understood. Upon colonization of the respiratory epithelial cells with *M. hyopneumoniae*, there is an infiltration of mononuclear cells (macrophages and lymphocytes) in the perivascular, peribronchial and peribronchiolar areas and alveolar septa, as well as hyperplasia of the lymphoid follicles around the airways, resulting in a chronic and prolonged inflammatory response (Sarradell et al., 2003). Thus, the interactions between the host's immune system and the mycoplasma are complex and ultimately determine resistance to infection and severity of disease (Simecka, 2005).

Innate immune response

Shortly after infection, mycoplasmas interact with the cells of the innate immune system such as phagocytes, macrophages and polymorphonuclear (PMN) cells. These cells are attracted by the release of immunogenic proteins and mediators during mycoplasmal infection (Sarradell et al., 2003). Histological lesions in the acute stage of *M. hyopneumoniae* infection are characterized by loss of respiratory cilia, exfoliation of ciliated cells, and accumulation of neutrophils and macrophages in and around airways. In the chronic stages, there is lymphoid hyperplasia or cuffing and thickening of the inter-alveolar septa (Goodwin, 1965).

The presence of inflammatory cytokines, the products of activated macrophages, suggests that mononuclear phagocytes, possibly alveolar macrophages, have an important role in the initiation of an inflammatory response. This can be histologically visible as soon as 7 days after infection (Choi et al., 2006). *M. hyopneumoniae* can directly stimulate macrophages to produce pro-inflammatory cytokines or other factors that have potential to modulate phagocytic activity. Even though macrophages are a first line of defense in the innate immune system, they become less effective upon *M. hyopneumoniae* infection (Thacker and Minion, 2010). For example, alveolar macrophages collected from pigs infected with *M. hyopneumoniae* had significantly lower phagocytic capacity than macrophages from uninfected animals (Caruso and Ross, 1990). This decreased phagocytic capacity is likely

important in the reduced clearance of *M. hyopneumoniae* as well as other secondary bacterial pathogens (Thacker and Minion, 2010).

Cytokines are vital components of both humoral and cell-mediated immune responses, and they exert their biological effects by binding to specific receptors on target cell membranes. Based on their properties, some cytokines can be generally classified as pro-inflammatory cytokines (represented by IL-1, IL-6, interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α)) or as anti-inflammatory cytokines (represented by IL-10 and transforming growth factor beta (TGF- β)). They are very important mediators in both lung defense and inflammation, but their overall positive or negative effect on the host tissue can be difficult to predict because of the complexity of both cellular and tissue environments (Yang et al., 2004).

The production of proinflammatory cytokines has been shown to be associated with the development of *M. hyopneumoniae* induced pneumonia. Increased levels of IL-1, IL-2, IL-6, IL-8, IL-12, IL-18, IFN- γ and TNF- α have been reported in *M. hyopneumoniae*-infected pigs (Asai et al., 1993; Asai et al., 1994; Choi et al., 2006; Lorenzo et al., 2006; Muneta et al., 2006; Redondo et al., 2009; Rodriguez et al., 2007; Rodriguez et al., 2004; Thacker et al., 2000a; Thanawongnuwech et al., 2004). Indeed, it is increasingly recognized that for many bacteria, induction of cytokines is a major virulence mechanism (Henderson et al., 1996). However, some mycoplasmas colonize the respiratory tract with no apparent clinical symptoms due to their capacity to downregulate NF κ B or to induce anti-inflammatory cytokines such as IL-10, IL-13, or TGF- β . This illustrates the complex network of synergistic and antagonistic influences induced by mycoplasmas on cells of the immune system (Rottem, 2003). Indeed, IL-10 levels are increased in the bronchoalveolar lavage (BAL) fluid 28 days after inoculation with *M. hyopneumoniae* (Thanawongnuwech and Thacker, 2003).

In addition, different *M. hyopneumoniae* strains may vary in their effect on the pig's immune system, resulting in variations in the inflammatory cell responses in the lungs. Meyns et al. (2007) showed that more leukocytes were found in the lung tissue and more IL-1 β and TNF- α were detected in BAL fluid after infection with a highly virulent (HV) strain compared to infection with a low virulent (LV) strain. It is possible that different strains of *M. hyopneumoniae* induce the production of cytokines to a different extent. The resulting inflammatory response will be determined by the intricate cross-talk of all cytokines involved, and this may lead to a different outcome for different strains (Razin et al., 1998).

Mycoplasma can stimulate different cell types through the interaction with toll-like receptors (TLR), which recognize pathogen associated molecular patterns (PAMPs). This

recognition leads to the activation or modulation of subsequent immune and inflammatory responses. The exact mechanisms are not fully understood, but porcine TLR2 and TLR6 will recognize lipoproteins from the mycoplasma and stimulate cells. There are indications that blocking TLR2 and TLR6, the overproduction of proinflammatory cytokines, such as TNF- α , may be regulated. In addition, that the vaccination may reduce the expression of TLR6 contributing to the prevention of mycoplasmal pneumonia in swine (Muneta et al., 2003; Regia Silva et al., 2011).

Adaptive immune response

T cells can be regarded as the key players of the adaptive immune system, serving as a link between the innate and adaptive immune system. Specific protective defense mechanisms consist of stimulation of cell-mediated immunity, opsonisation and phagocytosis of organisms and production of systemic as well as local anti-mycoplasmal antibodies. Antigen presenting cells (macrophages, dendritic cells, B cells) pick up the pathogens and subsequently process and present them to be recognized by pathogen-specific T cells. Once activated, T cells influence all arms of immunity. They are in charge of inducing and maintaining acquired immunity and generating memory lymphocyte populations (Jones and Simecka, 2003).

The role of the cell mediated immunity in animals with mycoplasmal pneumonia is ambiguous. Suppression of the T-cell response achieved by thymectomy or injection of antithymocyte serum before infection resulted in the development of less severe microscopic lesions of pneumonia compared to immunocompetent infected pigs. However, *M. hyopneumoniae* could spread systemically as it was re-isolated from the spleen of a thymectomized pig (Tajima et al., 1984). These results suggest that a T-cell-dependent mechanism may be important in the development of pneumonia but it is also important in preventing the invasion and systemic spread of *M. hyopneumoniae* (Thacker and Minion, 2010). Alterations to the cellular immune response clearly play an important role in the induction of the disease and the ability of the organism to persist in infected pigs.

M. hyopneumoniae is able to persist for a long time within infected pigs, possibly by suppressing the efficacy of the immune response or by evading it. During the progression of the disease, *M. hyopneumoniae*-specific and non-specific IFN- γ and IL-10 cytokine responses become inhibited (Muneta et al., 2008). Inhibition of IFN- γ production may enhance secondary viral infections such as PRRSV and swine influenza virus (Thacker et al., 1999; Yazawa et al., 2004). Inhibition of IL-10 may lead to over activation of macrophages and tissue damage because of its important anti-macrophage activity (Muneta et al., 2008).

Indeed, induction of IL-10 secretion by vaccination resulted in a lower influx of macrophages in the bronchoalveolar lymphoid tissue in vaccinated pigs compared to non-vaccinated animals, after experimental infection with *M. hyopneumoniae* (Vranckx et al., 2012).

Helper T cells (Th) are an important component in the response to mycoplasma respiratory disease and are the most numerous subset in the lymphoid infiltration. Although less numerous, cytotoxic T-cells are also observed in lung lesions caused by *M. hyopneumoniae* (Sarradell et al., 2003). Th-1 cells are responsible for activating and increasing phagocytic and cytotoxic activities of macrophages (Asai et al., 1994; Messier et al., 1990). Therefore, the presence of T-cells and overproduction of certain cytokines such as IL-1 and IFN- γ exacerbate the host mediated tissue damage.

Specific lung sections of pigs inoculated with *M. hyopneumoniae* revealed that cells producing IgM, IgA and IgG were present (Messier et al., 1990). In addition, particular increase in the level of IgG and IgA is found in tracheobronchial secretions, lungs and serum of naturally or experimentally infected pigs (Messier et al., 1990; Redondo et al., 2009; Rodriguez et al., 2004; Suter et al., 1985). IgG participates in opsonization and phagocytosis by alveolar macrophages, whereas IgA seems important in local immunity (Sarradell et al., 2003).

The induction of an immune response by *M. hyopneumoniae* is slow, with seroconversion taking at least 3-6 weeks to develop in experimentally infected pigs (Thacker et al., 2000b). No direct correlation has been found between the induction of serum antibodies and protection against *M. hyopneumoniae* (Djordjevic et al., 1997).

It is suggested that an effective immunity against *M. hyopneumoniae* requires humoral and cell-mediated immune responses (Haesebrouck et al., 2004; Thacker et al., 2000a). Both immune responses are driven by activation of CD4⁺ Th cells. Differentiation of naive CD4⁺ Th cells into Th1 and Th2 cells determines whether humoral or cell-mediated immunity will be predominant (Stevens et al., 1988). CD4⁺ Th1 cells are involved in the cell-mediated immune response and activate B cells to produce opsonizing antibodies, such as IgG2a, whereas CD4⁺ Th2 cells favour humoral immunity and secretion of IgG1 and IgA (Okamba et al., 2007).

Mucosal Immunity

Mucosal surfaces cover the largest surface area in the body and almost 80% of total immune cells in the body are localized in the mucosa-associated lymphoid tissues (MALT) and at mucosal sites. Nasopharyngeal MALT contains the entire repertoire of immune cells

which are strategically located to orchestrate regional immune functions against airborne infections (Mann et al., 2009).

Mucosal immunity is central to immune homeostasis and protection against the vast majority of pathogens that invade via mucosal surfaces. The particular hallmark of mucosal immunity remains the production and presence at the luminal mucosal surfaces of secretory IgA (S-IgA). The production of S-IgA consists of IgA class switching of activated B cells in the germinal centres of mucosal tissues and lymph nodes, and their dissemination via efferent lymphatics through the blood stream to mucosal tissues (Meeusen, 2011). In general, S-IgA has multiple roles in mucosal defense (Lamm, 1997). It promotes the entrapment of antigens or microorganisms in the mucus, preventing direct contact of pathogens with the mucosal surface, a mechanism that is known as 'immune exclusion'. Alternatively, S-IgA of the appropriate specificity might block or sterically hinder the microbial surface molecules that mediate epithelial attachment (Hutchings et al., 2004).

The role of local IgA in *M. hyopneumoniae* infection is not totally clear, but some studies found that IgA responses are, next to cell-mediated immunity, important for protection (Sarradell et al., 2003; Thacker et al., 2000a). *M. hyopneumoniae* is primarily found on the mucosal surface of the trachea, bronchi, and bronchioles (Blanchard et al., 1992), and the primary defense of this tissue is the mucosal immune system by the production of S-IgA, which prevents the interaction of the pathogens with receptors on the mucosal cell surface. A study investigating the effect of vaccination found that vaccination induced secretion of *M. hyopneumoniae*-specific antibodies into BAL fluid, suggesting that these antibodies may be important in the prevention or resolution of mycoplasmal pneumonia (Thacker et al., 2000b). Because BAL fluid is in close contact with *M. hyopneumoniae* organisms attached to the cilia of the respiratory tract, antibodies in this compartment may be an important mechanism of defense against clinical disease (Thacker et al., 2000a).

However, systemic immunisation with the currently available vaccines is not able to generate a full protective immunity (Meyns et al., 2006; Villarreal et al., 2011b). This partial protection could be due to parenteral administration of bacterins. It has been demonstrated that systemic stimulation of the immune system mainly results in systemic protection with low mucosal immune responses. Conversely, optimal stimulation of the mucosal immune system generates both mucosal and systemic immunity (Czerkinsky et al., 1999). The ideal vaccine would be mucosally administered and able to stimulate a suitable mucosal immunity, including specific Th response and IgA, which can prevent the adherence of pathogens to mucosal cell surfaces (Neutra and Kozlowski, 2006).

1.1.4 Lesions

Gross lung lesions following *M. hyopneumoniae* infections consist of purple to grey consolidated areas that are mainly located in the apical and cardiac lobes and the cranial parts of diaphragmatic lobes (Thacker and Minion, 2010) (Figure 2). Lesions appear from 7 days after experimental infection onwards and reach a maximal size at about 4 weeks after infection (Kobisch et al., 1993). The affected lung parts are characterized by a meaty aspect, while a catarrhal exudate is often present in the airways. The bronchial and mediastinal lymph nodes are usually enlarged (Goodwin, 1965).

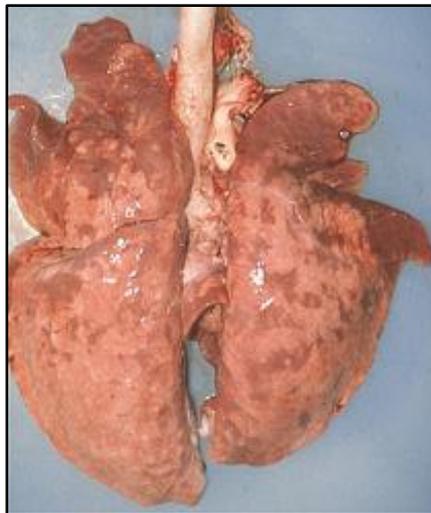


Figure 2: Lung of a *M. hyopneumoniae* infected pig.

Under field conditions, the clinical symptoms and lesions are often complicated by infections with other respiratory pathogens (Sibila et al., 2009). A larger portion of the lung may be affected, lesions can be more firm and heavy, and mucopurulent exudate can be expressed from the airways (Thacker and Minion, 2010). A correlation between the onset of clinical symptoms (coughing) and pneumonic lesions has been described (Kobisch et al., 1993). In the latter study, macroscopic lesions and coughing were seen 1 and 2 weeks post infection, respectively. Seven to nine weeks after infection, lesions were recovering and the coughing disappeared.

Although gross lesions are suggestive of EP, they are not pathognomonic for *M. hyopneumoniae*, as other organisms may produce similar lesions. In the presence of other respiratory pathogens, the lesions become more diffuse and they are sometimes difficult to

differentiate from those induced by other pathogens like swine influenza virus or the combination of *Pasteurella multocida* and Aujeszky's disease virus (Done, 1991; Fuentes and Pijoan, 1987). The lung consolidation areas regress by 2 to 4 months post-infection (Sorensen et al., 1997), but the organism can remain present in the lungs at least 6 months after infection (Fano et al., 2005).

Microscopically, pulmonary tissue modifications observed in pigs in the acute phase of infection consist of hyperplasia of the epithelial cells and an increased perivascular and peribronchiolar accumulation of mononuclear cells (Kobisch and Friis, 1996). In the early stages of the disease, there is a loss and exfoliation of cilia, and there are small accumulations of neutrophils in the lumina around the airways. Electron microscopy studies showed loss of cilia at 6-11 weeks post-infection (Blanchard et al., 1992) and the presence of mycoplasmas and leukocytes in affected tissue surface (Irigoyen et al., 1998). Lymphocytes and to a lesser extent also macrophages form cuffs around the airways. In more chronic lesions, lymphocytic cuffs are more prominent and may contain lymphoid nodules (Thacker and Minion, 2010). Lymphoid hyperplasia itself may be related to clinical signs, because pressure from aggregates of lymphoid tissue may obliterate the lumen of the soft-walled bronchioles and cause collapse of surrounding alveoli ("cuffing" pneumonia) (Baskerville, 1972).

1.1.5 Control measures

M. hyopneumoniae infections can be controlled by combining several factors such as the optimization of management practices, biosecurity measures and housing conditions, and the use of antibiotics and vaccination (Maes et al., 2008). The improvement of management practices is paramount to controlling the disease, and this is the first measure that should be taken once *M. hyopneumoniae* has been detected.

Antimicrobials such as tetracyclines and macrolides are often used to treat respiratory diseases such as EP (Vicca et al., 2004). Due to the localisation of the organism on the surface of the respiratory tract, only antimicrobials that are able to reach high concentrations in the mucus and the fluids of the respiratory tract are expected to be effective *in vivo*. Pigs treated with effective antibiotics have less clinical signs and lung lesions, and a lower number of secondary infections (Ciprian et al., 2012; Vicca et al., 2005). Despite the success of medication programmes in the control of *M. hyopneumoniae* infections, often only partial protection is achieved. The results may be inconsistent since outbreaks are possible when treatment is stopped (Thacker and Minion, 2010). Also, preventive or strategic medication of pigs with antimicrobials should be minimized as much as possible to avoid development of antimicrobial resistance.

Vaccination with inactivated, adjuvanted whole-cell bacterins (alone or in combination with antibiotics) is frequently used worldwide to control *M. hyopneumoniae* infections (Haesebrouck et al., 2004). The efficacy of vaccination has been demonstrated in many different studies. Although vaccination confers beneficial effects in infected pigs, these effects are variable between herds. The variability may result from different factors such as the infection level, the age of infection, complicating factors and the variability between different isolates of *M. hyopneumoniae* (Calus et al., 2007; Villarreal et al., 2011a). However, vaccination is still considered the most effective practice for controlling this infection (Mateusen et al., 2002). The major advantages of *M. hyopneumoniae* vaccination include improvement of the daily weight gain (2-8%) and the feed conversion ratio (2-5%), a shorter time to reach slaughter weight, reduced clinical signs, lung lesions and less treatment costs (Maes et al., 1998; Maes et al., 1999).

1.1.6 Vaccines

1.1.6.1 Commercial Vaccines

Vaccination with commercial bacterins has been an important tool to control infection by *M. hyopneumoniae* worldwide. Despite the beneficial effects, these bacterins provide only partial protection and do not prevent the colonization of *M. hyopneumoniae* on the epithelial cells (Thacker et al., 2000a). Some studies indicate that currently available commercial vaccines can reduce the number of organisms in the respiratory tract (Meyns et al., 2006; Vranckx et al., 2012), and consequently, the infection level in the animals, and by extension likely also in the herd (Sibila et al., 2007). Although vaccination significantly reduces clinical symptoms and lung lesions, only a limited reduction occurs in the transmission of *M. hyopneumoniae* (Meyns et al., 2006; Villarreal et al., 2011b). These findings reinforce the idea that vaccination alone is not sufficient to eliminate *M. hyopneumoniae* in infected pig herds. To achieve desirable results, vaccination should be practiced in combination with other management factors controlling the infection (Haesebrouck et al., 2004).

Despite the fact that vaccination against *M. hyopneumoniae* is widely practiced, many aspects about its effect on transmission of the pathogen, effect on different strains and on the immunological response remain unclear and need to be further investigated. Nevertheless, studies suggest that mucosal antibodies and systemic immune responses are important for the control of this disease (Thacker et al., 2000a). The commercial vaccines induce the production of specific antibodies in serum, although the antibody response may differ between the vaccines (Thacker et al., 1998), and the seroconversion rate among animals can range from 30% – 100% (Sibila et al., 2004). The correlation between the induction of specific antibodies and protection against pneumonia is unclear. Challenge experiments in pigs have shown that the concentrations of antibodies in serum are not correlated with protection against *M. hyopneumoniae* (Djordjevic et al., 1997). Thus, the presence and concentration of antibodies in serum do not appear to be the best way to evaluate protective immunity.

Few studies have evaluated the effects of vaccination of the commercial bacterins on mucosal immune system and cell mediated immunity. Thacker et al. (2000a), evaluating BAL fluid and lymphocytes isolated from blood, indicated that a commercial vaccine induces a local mucosal and systemic cell mediated immune response detected by ELISpot and ELISA. However, the vaccination failed to induce nonspecific changes in the peripheral CD4⁺ and CD8⁺ cells, analyzed by flow cytometry. In contrast, differences between vaccinated and

control pigs in CD4⁺ and CD8⁺ cells were detected in a study performed by Kick et al. (2011). This indicates that there is considerable variation between published reports and that little is known about the exact mechanism of protection of the commercially available *M. hyopneumoniae* vaccines.

All commercially available *M. hyopneumoniae* vaccines are made from killed mycoplasma cultures (bacterins) but have different adjuvants. Some adjuvants are considered relatively mild, like aluminium salts, whereas the oil-based ones, usually in the form of emulsions, are more reactive and may take a longer time to be taken up, therefore giving a more prolonged release and stimulation (Yuki and Kiyono, 2003). The difference in adjuvant composition in commercial vaccines might also influence the type of induced immune response and the levels of protection.

Besides this, the current commercial vaccines used to control enzootic pneumonia are mostly based on the J-strain, which was originally isolated from a pig herd in the UK (Goodwin and Whittlestone, 1963). The variable results observed in the vaccine efficacy under field conditions may be due, among other factors, to antigenic differences between the strains circulating in pig herds and the vaccine strain.

The role of maternal immunity in protection of newborn piglets is not entirely clear and the potential interference with development of active immunity needs to be better understood. It has been demonstrated that lymphocytes that were passively transferred from vaccinated dams to their offspring in colostrum were able to proliferate and participate in a functional response to *M. hyopneumoniae* (Bandrick et al., 2008). Also, it has been shown that passive immunity induced by sow vaccination may be consistent with early vaccination to *M. hyopneumoniae* in piglets (Martelli et al., 2006). One study reported that a single dose of *M. hyopneumoniae* bacterin to pigs of approximately 1 week of age induced long-lasting protective immunity, with reduction in the extent of lung lesions after challenge with a virulent strain of *M. hyopneumoniae* (Reynolds et al., 2009). An early vaccination regimen should induce immunity around the time of weaning, when pigs from different litters are mixed together, and are likely to face their first infectious challenge, and when the risk of developing multi-factorial severe pneumonia is exacerbated (Martelli et al., 2006). Meyns et al. (2004) and Villarreal et al. (2011b) estimated under experimental and field conditions, respectively, that one infected animal will infect an average of one susceptible penmate during a nursery period of 6 weeks. These results emphasize the importance of the implementation of control strategies from an early age onwards.

Different strategies of vaccination have been adopted depending on the needs of a particular herd and variables such as the production system, type of herd, infection pattern and preferences of the pig producer. The decision to vaccinate or not depends on the economic feasibility in the farm and the profits that the vaccination will bring in relation to the economic losses caused by the disease (Maes et al., 2008).

In table 1, a description of some commercially available vaccines against *M. hyopneumoniae* licensed in Europe is given. Commercial names, antigen and adjuvant constitution are presented, as well as the indications of administration.

Table 1: Commercially available *M. hyopneumoniae* bacterins licensed in Europe (2013)

Vaccine	Strain	Adjuvant	Route of administration	Age of administration	Boosts needed
HYORESP (Merial)	NI*	Aluminium hydroxide	IM	>5 days	3-4 weeks
INGELVAC MYCOFLEX (Boehringer Ingelheim)	J strain isolate B-3745	Impran (water- in-oil adjuvant emulsion)	IM	>21 days	-
M+Pac (Intervet Int.)	NI*	Aluminium hydroxide	IM	>7 days	3-4 weeks
MYPRAVAC SUIS (Hipra Lab)	J strain	Levamisole and carbomer	IM	7-10 days	3 weeks
PORCILIS M. HYO (Intervet)	Strain 11	dl- α -tocopherol acetate	IM	7 days	3 weeks
STELLAMUNE MYCOPLASMA (Eli Lilly)	NL 1042	Mineral oil and lecithin	IM	7 days	2-4 weeks
STELLAMUNE ONCE (Eli Lilly)	NL 1042	Amphigen Base, and Drakeol 5 (mineral oil)	IM	7 days	-
SUVAXYN M.HYO (Pfizer A.H.)	P-5722-3	Carbopol	IM	7 days	2 weeks
SUVAXYN MH- ONE (Pfizer A.H.)	P-5722-3	Carbopol and squalane	IM	7 days	-
SUVAXYN M.HYO PARASUIS (Pfizer A.H.)	P-5722-3	Carbopol	IM	7 days	2 weeks

*No information available

1.1.6.2 Development of new generation vaccines against *M. hyopneumoniae*

In recent years, there has been a remarkable expansion of new approaches to identify relevant antigenic components for use in vaccines, and these new techniques are beginning to be applied in the development of veterinary vaccines (Movahedi and Hampson, 2008). Progresses in microbiology, molecular biology, immunology and bioinformatics have led to a more rational approach in vaccine development (Haesebrouck et al., 2004). For many diseases, this new approach can be an alternative to replace the traditional method of vaccine preparation, which follows the principles established by Pasteur (Rappuoli, 2001).

With recombinant DNA technology, the "second generation" vaccines emerged based on the use of recombinant proteins. More recently, the availability of complete genome sequences have led to a new approach in vaccine development (Scarselli et al., 2005), called reverse vaccinology (Rappuoli, 2001). The reverse vaccinology approach starts from the genomic sequence and, by computer analysis, predicts those antigens that are most likely to be vaccine candidates. The approach can, therefore, be very simple, and poses the question of whether any of the potential antigen candidates can provide protective immunity (Rappuoli, 2001). This has led to a "third generation" of vaccines (Movahedi and Hampson, 2008).

The combination of *in silico* analysis of whole genome sequences and recombinant DNA technology has several advances in the development of third generation vaccines (Movahedi and Hampson, 2008). Through computational analysis, virtually all proteins that the pathogen can express both *in vivo* and *in vitro* can be selected, without prior knowledge of their abundance, immunogenicity or expression during infection (Movahedi and Hampson, 2008). After selecting a group of genes of the genome, the next steps include: cloning and expression of selected genes, purification of recombinant proteins to identify potential candidates for protection (Grandi, 2001). Advantages of recombinant subunit vaccines are numerous. First of all, the pathogen can be entirely excluded from the production of the vaccine, which eliminates the risks associated with contamination by toxic compounds, and reversion to virulence. Besides, the possibility of optimizing the presentation forms of these antigens (Liljeqvist and Stahl, 1999).

However, one of the major disadvantages is that the reverse vaccinology can be applied only for the discovery of protein antigens and not for other antigens like lipopolysaccharides and glycolipids, which are important components of many successful vaccines. In addition, the feasibility of the approach relies heavily on the availability of a high-throughput system to screen protective immunity. Unfortunately, the limited knowledge of vaccine immunology

makes it difficult to find correlations with protection and, therefore, screening for protective immunity is the rate-limiting step of reverse vaccinology (Rappuoli, 2001; Serruto and Rappuoli, 2006). Besides, the necessity of having the publication of the complete genome sequence of many bacteria, parasites and viruses.

The genome sequencing of four *M. hyopneumoniae* strains (Liu et al., 2011; Minion et al., 2004; Vasconcelos et al., 2005), has allowed the rational choice of sequences that encode proteins that might be used for the development of a protective vaccine and for use in diagnostic tests of EP. The small genome of this agent, as well as, the limited number of secreted or surface proteins favours the use of reverse vaccinology.

The technology of recombinant DNA associated with the approach of the reverse vaccinology have provided valuable tools for the production of *M. hyopneumoniae* recombinant proteins in heterologous systems. The recombinant subunit vaccines are based on fractions of the organism and are produced using heterologous protein expression systems.

The heterologous recombinant proteins can be produced in both eukaryotic and prokaryotic organisms and, once expressed, can be readily purified and administered to animals in high concentrations (Clark and Cassidy-Hanley, 2005). *E. coli* is the most attractive system for production of recombinant proteins (Baneyx, 1999). However, despite the fact that genetic characteristics of *E. coli* are well known, not all genes can be efficiently expressed in this microorganism. An example is the expression of genes from *M. hyopneumoniae* containing codons TGA. TGA encodes the amino acid tryptophan in Mycoplasma, while it is a stop codon in other organisms. This feature has hindered the expression of these genes in *E. coli*. Mutations capable of replacing TGA by TGG have been the strategy used to circumvent this problem. Simionatto et al. (2009) described the optimization of a PCR protocol for site directed mutation in codon TGA genes from *M. hyopneumoniae*, obtaining satisfactory results in the expression of these genes in *E. coli*.

Constant effort is being directed towards the investigation of new vaccines that may offer a better protection against *M. hyopneumoniae* infections. Several studies have evaluated recombinant proteins of *M. hyopneumoniae*, in various forms of administration and formulations. Some of them were evaluated individually (Galli et al., 2012; Simionatto et al., 2012), others were associated with attenuated bacterial or viral vectors (Chen et al., 2006a; Chen et al., 2006b; Chen et al., 2001; Fagan et al., 2001; Okamba et al., 2010; Okamba et al., 2007; Shimoji et al., 2003; Zou et al., 2011), fused to mucosal adjuvants (Conceição et al., 2006), and also evaluated as a pool of antigens (Chen et al., 2008). Most of these recombinant proteins were evaluated only in mice. Only a few of them were tested in challenge

experiments in pigs. Differences in the immunity induced by these antigens were observed, which might be due to differences in the vaccine constructs, the route of immunization, the correct folding and/or other post-translational modification that may contribute to the ability to generate antibodies by the antigens (Pinto et al., 2007a). These evaluations suggest that these new vaccine approaches may represent promising strategies, and may be economically feasible to control EP. Table 2 summarizes the antigens, adjuvants, vectors and routes of immunization used in the studies on experimental *M. hyopneumoniae* vaccines so far.

Table 2: Overview of experimental vaccines against *M. hyopneumoniae*

Antigen	Kind of vaccine	Vector/Adjuvant	Species	Route	Challenge infection	Reference
P97	Recombinant Subunit	Freund's	pig	IM**	yes	King et al., 1997
NrdF (R2)	Recombinant Vector	<i>Salmonella</i> Typhimurium aroA SL3261	mice	Oral	no	Fagan et al., 1997
P97 (R1)	Recombinant Vector	Pseudomonas exotoxin A	mice and pig	SC*** and IM	no	Chen et al., 2001
NrdF (R2)	Recombinant Vector	<i>Salmonella</i> Typhimurium aroA SL3261	pig	Oral	yes	Fagan et al., 2001
P42	DNA	pcDNA3	mice	IM	no	Chen et al., 2003
P97 (R1R2)	Recombinant Vector	<i>Erysipelothrix rhusiopathiae</i> YS-1	mice and pig	SC and IN****	no	Shimoji et al., 2003
P97 (R1)	Recombinant Vector	<i>Salmonella</i> Typhimurium aroA CS332	mice	Oral	no	Chen et al., 2006a
NrdF (R2)	Recombinant Vector	<i>Salmonella</i> Typhimurium aroA CS332	mice	Oral	no	Chen et al., 2006b
P97 (R1)	Recombinant Subunit	LTB	mice	IM and IN	no	Conceição et al., 2006
P97 (R1)	Recombinant Vector	Adenovirus	mice	IM and IN	no	Okamba et al., 2007
P97 (R1R2)	Recombinant Vector	<i>Erysipelothrix rhusiopathiae</i> Koganei	pig	Oral	yes	Ogawa et al., 2009
P97 (R1)	Recombinant Vector	Adenovirus	pig	IN	yes	Okamba et al., 2010
P36	Recombinant Vector	<i>Actinobacillus pleuropneumoniae</i> SLW36	mice	IM	no	Zou et al., 2011
34*	Recombinant Subunit	Aluminun	mice	IM	no	Simionatto et al., 2012
P37, P42, P46, P95	Recombinant Subunit and DNA	Aluminun and pcDNA3	mice	IM	no	Galli et al., 2012

* 34 previously uncharacterized recombinant proteins of *M. hyopneumoniae* were evaluated in this study.

** Intramuscularly

*** Subcutaneously

**** Intranasal

The P97 protein, which was identified as an important adhesin of *M. hyopneumoniae* (Zhang et al., 1995) has been the most studied and best defined potential protective antigen. It has been evaluated in several studies using different vaccine formulations. King et al. (1997) reported that a subunit vaccine based on recombinant adhesin P97 did not induce significant protection in challenged pigs. However, when the C-terminal region of P97 (R1) was fused to *Pseudomonas* toxin A, immunized mice and pigs produced specific immune responses against R1 (Chen et al., 2001). Oral vaccination with recombinant *E. rhusiopathiae* strains expressing the P97 protein reduced the severity of pneumonic lung lesions caused by *M. hyopneumoniae* infection, showing that *E. rhusiopathiae* may be a promising vaccine vector for delivering foreign antigens to the immune systems of pigs (Ogawa et al., 2009).

Apart from the antigen in the vaccine, also the adjuvant and the route of immunization play a role in protection. Mucosal adjuvants and intranasal vaccine delivery have received special attention as alternatives that may increase the mucosal immunity. Conceição et al. (2006) have reported that a recombinant subunit vaccine containing the R1 subunit of P97 fused to the B subunit of thermolabile enterotoxin of *E. coli* (rLTBR1), a parenteral and mucosal adjuvant, induced systemic and mucosal antibodies against R1 in mice. Shimoji et al. (2003) reported that a YS-19 attenuated strain of *Erysipelothrix rhusiopathiae* expressing the R1 region of P97 was able to reduce lung lesions in intranasally (IN) immunized pigs when they were challenged. However, there was no humoral or cell-mediated immune response. Further, Okamba et al. (2007) showed that mice immunized IN with defective adenovirus expressing the C-terminal region of P97 (rAdP97c) induced a systemic Th1/Th2 immune response and local immunity. This same construct was evaluated in pigs in comparison with a commercial vaccine. rAdP97c significantly reduced the severity of the inflammatory response and the amount of *M. hyopneumoniae* in the respiratory tract. Also the average daily weight gain was slightly improved. However, the commercial vaccine was more effective in inducing protective immunity (Okamba et al., 2010). These results suggest that the kind of immunity induced by this antigen may be influenced by differences in the vaccine construct, the route of immunization, and the adjuvant or vector used.

Mice immunized orally with *Salmonella* Typhimurium aroA CS332 harboring the R1 region of P97 (Chen et al., 2006a) and the R2 subunit of ribonucleotide reductase (NrdF) of *M. hyopneumoniae* (Chen et al., 2006b) showed a Th1-based immune response, but no humoral or mucosal immune responses. However, when the NrdF R2 subunit was expressed in *Salmonella* Typhimurium aroASL3261, a mucosal immune response was observed in mice

(Fagan et al., 1997). When used for immunizing pigs, this antigen was able to reduce lung lesions caused by an experimental *M. hyopneumoniae* infection (Fagan et al., 2001).

Apart from recombinant subunit vaccines that have been evaluated in mice and pigs, immune responses to DNA vaccines in mice have also been investigated. DNA vaccines based on heat shock protein P42 induced both Th1 and Th2 immune responses in mice. In addition, antiserum from the immunized animals was found to inhibit the growth of *M. hyopneumoniae in vitro* (Chen et al., 2003). Similar results were described using a DNA vaccine with the antigen P46. Both Th1 and Th2 immune responses were obtained in mice, with an increase in the IFN- γ level (Galli et al., 2012). Thus, DNA vaccines might represent a promising strategy for control of EP.

Despite the immunizing properties of the recombinant antigens and DNA vaccines tested so far, none of them was able to induce a protection as good as the commercial vaccines. Multivalent formulations could be another alternative in offering better protection against *M. hyopneumoniae* (Chen et al., 2008). Five recombinant antigens of *M. hyopneumoniae* (P97, P97R1, NrdF, P36, and P46) were evaluated as DNA and protein vaccines. The analysis of mice immunized with the cocktail of antigens administered as DNA vaccine revealed that only P97 and P36 induced IgG in the serum. Intramuscular immunization with a cocktail of antigens (P97, P97R1, NrdF, P36, and P46) as a DNA vaccine induced a Th1 immune response, while antibody responses appeared to be antigen dependent. Subcutaneous immunization with the same cocktail administered as a recombinant subunit vaccine induced humoral and Th1 immune responses. When a combination of DNA and subunit vaccine was used, both humoral and Th1 responses were obtained. In addition, P97 was not recognized by serum antibodies from mice immunized with a commercial bacterin, which may be an indication of the lack of expression of this antigen in inactivated whole-cell vaccines (Chen et al., 2008).

The identification and characterization of immunogenic *M. hyopneumoniae* proteins is an important step towards the development of improved vaccines. Despite the difficulties due to the unusual codon of mycoplasmas (Razin et al., 1998), Simionatto et al. (2009; 2010) have previously reported the cloning and expression of a high number of *M. hyopneumoniae* recombinant proteins in *E. coli*. Antigenic and immunogenic properties of these proteins were analysed. Some of them were evaluated in mice and showed to be potential as vaccine candidates against *M. hyopneumoniae*. Although the immune response of mice cannot be extrapolated to other species, the results warrant further investigation with promising proteins as subunit vaccines in challenge experiments in pigs (Simionatto et al., 2012).

Most of these experimental vaccines must be evaluated in pigs under both experimental and field conditions. To date, few formulations have been assessed in *M. hyopneumoniae* challenge experiments in pigs (Fagan et al., 2001; Ogawa et al., 2009; Okamba et al., 2010), and these conferred only partial protection with some reduction of pulmonary lesions.

1.1.6.3 Mucosal Adjuvants: Immunomodulation by Bacterial Enterotoxins

Taking into consideration the above discussion, it is clear that we require a safe and effective vaccine against *M. hyopneumoniae*. Considering that genetically engineered vaccines composed of a single recombinant antigen, particularly subunit vaccines are poorly immunogenic, it is important to increase their immunogenicity by combining them with appropriate adjuvants (Soria-Guerra et al., 2011).

Currently used adjuvants such as aluminum hydroxide or phosphate have a demonstrated safety profile of many decades and their role in the implementation of successful vaccine programmes is impressive. However, these adjuvants have been associated with severe local reactions, such as erythema, subcutaneous nodules and contact hypersensitivity (Baylor et al., 2002). Oil emulsions of various compositions are also widely used as adjuvant in *M. hyopneumoniae* vaccines. They have been considered as potent adjuvants even though they have a tendency to induce granulomas at the injection sites (Aucouturier et al., 2001).

Inducing mucosal immunity with toxin-derived immune adjuvants has received a lot of attention, especially for vaccine development for bacterial pathogens. As *M. hyopneumoniae* infection starts in the respiratory tract, the ideal vaccine would be mucosally administered and able to stimulate a suitable mucosal immunity, including specific T helper (Th) response and immunoglobuline A (IgA), which can prevent the adherence of pathogens to mucosal cell surfaces (Neutra and Kozlowski, 2006).

One strategy has been to employ bacterial enterotoxins such as cholera toxin (CT) from *Vibrio cholera* or the related subunit B of heat-labile enterotoxin of *Escherichia coli* (LTB), a non-toxic subunit. These have been shown to be highly immunogenic, demonstrate potent immune-modulatory activity with a range of antigens either fused or co-administered to them, in different animal models, enhance immunogenicity as well as to confer protective efficacy (Conceição et al., 2006; da Hora et al., 2011; Grassmann et al., 2012; Qiao et al.,

2009; Zhou et al., 2009). They are considered as potent mucosal and parenteral adjuvants (Cox et al., 2006; da Hora et al., 2011; Simmons et al., 2001), enhancing both mucosal IgA as well as systemic antibody responses.

In developing veterinary mucosal vaccines and vaccination strategies, mucosal adjuvants are one of the key players for inducing protective immune responses. The biological effects of CT and LTB are mediated by the binding and internalization of the molecules into mammalian cells. The principle receptor for both is GM1-ganglioside, a glycosphingolipid found ubiquitously on the surface of all cells, including enterocytes, dendritic cells, macrophages, B and T lymphocytes (Cox et al., 2006).

Although the mechanisms by which the enterotoxin-based adjuvants exert immunomodulating effects are not well characterized, enhancement of inflammatory cytokine and chemokine production and transient recruitment of immune effector cells to the site of immunization are likely involved (Ryan et al., 2000). LTB is also known to influence the dendritic cell maturation (Petrovska et al., 2003), antigen presentation and T-cell activation and promote the induction of antigen-specific cytotoxic T lymphocyte responses in mouse models (Fu et al., 2009). The use of LTB as an adjuvant has resulted in balanced cytokine response, involving the production of both Th1 and Th2 cytokines (Fromantin et al., 2001).

The competence of LTB to modulate or improve vaccine-induced immune responses appears to depend to some extent on the antigen-LTB combination (there are difference in response for the B subunit whether it is used as a mixture or coupled to the antigen), the vaccination route and the dose (da Hora et al., 2011). The route of administration appears to be a critical factor influencing the immune-enhancing activities of LTB. Although LTB has demonstrated potent mucosal adjuvant activity when administered intranasally or orally (Fingerut et al., 2006; Richards et al., 2001; Yamanaka et al., 2006), it also stimulated a strong systemic and secretory response when administered parenterally (Fischer et al., 2010).

Bacterial enterotoxins as LTB have provided important insights into immunological mechanisms associated with mucosal adjuvant activity and significant practical benefits may follow in the form of new mucosal vaccines against *M. hyopneumoniae*. Conceição et al. (2006) have shown that immunization of mice with pure recombinant R1 of P97 adhesin did not induce specific systemic and mucosal antibodies to R1. In contrast, immunization with the R1 region fused to the B subunit of the heat-labile enterotoxin B subunit of *E. coli* (rLTBR1) produced high levels of specific antibody and cellular responses. However, the efficacy of this vaccine has not been tested in swine.

Despite the advantage of mucosal vaccinations for the prevention of *M. hyopneumoniae*, the number of experimental vaccines evaluating this route of immunization is still very limited. This is mainly due to important difficulties that are encountered by using the mucosal route such as:

- (1) the antigen should pass the mucosal barrier in sufficient amount,
- (2) mucosal tolerance mechanisms should be overcome,
- (3) protective immune mechanisms should be activated and
- (4) minimal/or no influence on mucosal functionality should occur.

Mucosal adjuvants are crucial in reaching these goals (Cox et al., 2006). However, *E. coli* heat-labile toxin (LT) has also been shown to have negative side effects in humans, as both native and mutant LT (mLT) used as adjuvants were recently associated with the development of facial nerve paralysis following intranasal delivery in humans (Lewis et al., 2009). It is, therefore, evident that these toxin adjuvants exhibit safety concerns that will likely prevent their use in nasally delivered vaccines for humans. Moreover, the B subunits of CT (CTB) and LT (LTB), which are not toxic, have been shown to promote tolerance to heterologous antigens. CTB has been the most studied, especially as conjugates with antigens. It has been tested in clinical assays for possible prevention of auto-immune and allergic diseases. Most of the studies of CTB conjugate with antigens showed to induce protective regulatory T cells in animal models of auto-immune or inflammatory diseases (Basset et al., 2010). However, the adjuvant activity of the B subunit has been a source of controversy and the many questions about the effects of these adjuvants remains confusing.

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CHAPTER 2.

AIMS OF THE STUDY

2. Aims of the study

Mycoplasma hyopneumoniae is the primary pathogen of enzootic pneumonia, a chronic respiratory disease that is widespread and causes major economic losses to the swine industry worldwide. Control measures include the optimization of management and housing conditions, the use of strategic medication and/or vaccination. However, the current control measures are beneficial from an economic point of view, but they do not provide a sustainable control of the disease. They cannot prevent colonization of *M. hyopneumoniae* in the respiratory tract, and do not significantly reduce the transmission of the pathogen. Also little is known about the exact mechanisms of the partial protection they induce.

The general aims of this thesis were, therefore, to assess the mode of the action of an existing commercial vaccine on the one hand and to develop and evaluate new recombinant vaccines against *M. hyopneumoniae* on the other hand, looking for more effective strategies for disease control. For development of the recombinant vaccines, two types of antigens were considered. One vaccine was based on proteins known to play a role in the adhesion and that were able to induce a partial protection against *M. hyopneumoniae* infection when evaluated individually. The other antigens were a group of transmembrane proteins (not previously characterized). The identification of these novel antigens might contribute to the development of improved vaccines.

The specific objectives were:

1. To analyze the systemic as well as the local mucosal immune response induced by intramuscular administration of a commercial *M. hyopneumoniae* vaccine in pigs.
2. To produce a recombinant chimeric protein composed of the P97R1, P42 and NrdF antigens of *M. hyopneumoniae* fused to LTB and to evaluate the capacity of this chimeric protein to induce a specific immune response in mice and pigs.
3. To identify, clone and characterize transmembrane proteins from *M. hyopneumoniae* which may be useful in vaccination against enzootic pneumonia.



CHAPTER 3.

EXPERIMENTAL STUDIES

3.1 Local and systemic immune responses in pigs intramuscularly injected with an inactivated *Mycoplasma hyopneumoniae* vaccine

3.2 Immune response induced by a recombinant chimeric protein containing *Mycoplasma hyopneumoniae* antigens fused to the B subunit of *Escherichia coli* heat labile enterotoxin LTB

3.3 Production and characterization of recombinant transmembrane proteins from *Mycoplasma hyopneumoniae*

CHAPTER 3.1:

Local and systemic immune responses in pigs intramuscularly injected with an inactivated *Mycoplasma hyopneumoniae* vaccine

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Abstract

The immune response induced by intramuscular administration of a commercial inactivated *Mycoplasma hyopneumoniae* whole-cell vaccine (Suvaxyn®MH One) was investigated in conventional *M. hyopneumoniae*-free pigs. The animals were assigned randomly to two groups: non-vaccinated and vaccinated. Pigs in the vaccinated group were injected intramuscularly with the vaccine at 7 days of age, whereas non-vaccinated pigs received physiological saline solution (PBS). Pigs were euthanized and necropsied at 30, 36 and 58 days of age. Blood, bronchoalveolar lavage (BAL) fluid, spleen, lung and bronchial lymph nodes (BLN) were collected. Serum and BAL fluid were tested for the presence of antibodies by ELISA. Monomononuclear cells from the peripheral blood and tissues were isolated to quantify the T cell subsets by flow cytometry, and cytokine production by ELISpot and ELISA. Antibodies against *M. hyopneumoniae* were detected in serum of most vaccinated pigs at 30 days of age. *M. hyopneumoniae* specific IgG, IgM and IgA were detected in BAL fluid from vaccinated animals, but not from control animals. Significantly higher numbers of IL-12 secreting cells were observed in the lung at day 58 in the vaccinated than in the non-vaccinated group ($p<0.05$). The number of IL-10 secreting cells from BLN was also higher in the vaccinated group at day 58 ($p<0.05$). After restimulation in vitro, lymphocytes from BLN and lungs secreted significantly higher levels of IL-12 in the vaccinated group at day 58. These results show that the vaccine induced both systemic and mucosal cellular and humoral immune responses.

Keywords: *Mycoplasma hyopneumoniae*; enzootic pneumonia; vaccine; immune response

Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary pathogen of enzootic pneumonia (EP). The disease occurs worldwide and causes important economic losses to the pig industry (Maes et al., 2008). Optimization of housing and management procedures combined with vaccination are the most effective tools to control EP (Lin et al., 2003; Maes et al., 2008). The currently used vaccines consist of adjuvanted, inactivated, whole-cell preparations and have been proven to be effective in reducing the clinical symptoms and lung lesions, and in decreasing performance losses associated with *M. hyopneumoniae* infections (Maes et al., 2008). They are very frequently used worldwide in commercial pig herds.

The commercial vaccines induce specific antibodies in serum, although no direct correlation has been found between the induction of antibodies and protection against *M. hyopneumoniae* (Djordjevic et al., 1997). Some studies suggest that mucosal antibodies and cellular immune responses might be important for the control of this disease (Thacker et al., 2000).

Only in few studies the effects of vaccination on mucosal and cell mediated immunity have been evaluated. Studies on bronchoalveolar lavage (BAL) fluid and lymphocytes isolated from blood evaluated by ELISpot and ELISA showed that a commercial inactivated vaccine induces mucosal and systemic cell mediated immune response. However, the vaccination failed to induce changes in the peripheral CD4⁺ and CD8⁺ cells population (Thacker et al., 2000). In contrast, differences between vaccinated and control pigs in the percentage of these cell populations, were detected in a study performed by Kick et al. (2011). The considerable variations between published reports demonstrate that the exact mechanism of protection induced by inactivated *M. hyopneumoniae* vaccines is not known.

The aim of this study was, therefore, to analyze the systemic as well as mucosal immune responses induced by intramuscular vaccination of pigs with a commercial *M. hyopneumoniae* inactivated whole-cell vaccine that had previously been shown to be effective in reducing disease signs and lung lesions associated with *M. hyopneumoniae* infection (Vraa-Andersen and Christensen, 1993).

Materials and methods

Experimental design

The study was performed after approval by the Ethical Committee for Animal Experiments of the Faculty of Veterinary Medicine, Ghent University (approval number EC2011/038). Thirty-three cross-bred healthy piglets free of *M. hyopneumoniae* and PRRSV

were used in this study. All animals were obtained from a herd that has been free of *M. hyopneumoniae* and PRRSV for more than 15 years based on repeated serological testing and absence of clinical symptoms and pneumonia lesions. At one week of age, blood for serology was collected from all animals. Thereafter, the piglets were randomly allocated to two different groups. At 7 days of age, 17 pigs of the vaccinated group were intramuscularly injected with 2 ml of the commercial *M. hyopneumoniae* vaccine Suvaxyn®MH One, Pfizer Animal Health, and 16 pigs of the control group were intramuscularly injected with 2 ml of phosphate buffered saline (PBS). The piglets were weaned at 24 days of age, transported to the Faculty of Veterinary Medicine, Ghent University, where they were necropsied at 30, 36 and 58 days of age. All piglets were euthanized by deep anesthesia with 0.3 ml/kg of a mixture of Xylazin (Xyl-M 2%®, VMD, Arendonk, Belgium) and Zolazepam and Tiletamine (Zoletil® 100, Virbac, Louvain la Neuve, Belgium), followed by exsanguination. At necropsy, lungs were macroscopically examined for the presence of lesions and blood and tissue samples from the spleen, the bronchial lymph nodes (BLN) and lungs were taken from all pigs. The lungs were removed and bronchoalveolar lavage (BAL) fluid was collected from the right lung. Blood was collected and part of it was used for serology. The other part was mixed with heparin (1:1000) and used for isolation of peripheral blood monomorphonuclear cells (PBMCs).

Nested and quantitative PCR (qPCR) for detection of *M. hyopneumoniae* DNA

DNA was extracted from the BAL with the QIAGEN protocol (QIAGEN, DNeasy Blood & Tissue kit, Belgium). For detection of *M. hyopneumoniae* DNA, both a nested and qPCR were performed as described previously (Marois et al., 2010; Stark et al., 1998), in order to avoid false negative results.

Isolation of monomorphonuclear cells from blood and tissues

PBMCs were isolated by density gradient centrifugation on Lymphoprep™ (Axis-Shield, Oslo, Norway). After lysis of erythrocytes in ammonium chloride (0.83%) and subsequent centrifugation, the pelleted cells were washed, resuspended in PBS with 1 mM of EDTA and counted.

After euthanasia, monomorphonuclear cells were isolated from the lungs, the BLN and the spleen. The monomorphonuclear cells were isolated by tearing the tissue apart, followed by lysis of erythrocytes with 0.15 M ammonium chloride. After centrifugation, the pelleted cells were washed, resuspended in leukocyte medium and counted (Van den Broeck et al., 1999).

Detection of *M. hyopneumoniae*-specific antibodies

Serum collected from the pigs at the day of vaccination and at necropsy was analysed for the presence of antibodies against *M. hyopneumoniae* using a blocking ELISA (IDEIA™ *Mycoplasma hyopneumoniae* EIA kit, Oxoid Limited, Hampshire, UK).

To determine the isotype of *M. hyopneumoniae*-specific antibodies in serum, peroxidase labeled goat anti-porcine IgA, IgG and IgM polyclonal antibodies (Bethyl Laboratories, Texas, TX, USA) and *M. hyopneumoniae* antigen coated microtitre plates from the IDEXX Mhyo ELISA (IDEXX, Hoofddorp, The Netherlands) commercial kit were used.

Previous experiments have shown that no reaction can be detected in BAL fluid using plates from the IDEXX kit which was developed for detection of antibodies in serum, so plates were coated with Tween®20 extracted *M. hyopneumoniae* antigens (Bereiter et al., 1990). BAL fluid samples were assayed for IgG, IgM and IgA antibodies against *M. hyopneumoniae* using peroxidase labeled goat anti-porcine IgA, IgG and IgM polyclonal antibodies (Bethyl Laboratories). The BAL fluid was tested undiluted. OD at 450 nm was measured and the mean OD value of the serum from the non-vaccinated animals plus two times the standard deviation was used as cut-off value to determine the number of positive animals in the vaccinated group for each immunoglobulin. Values equal to or higher than the cut-off were considered as positive.

Determination of T cell populations in different tissues

Flow cytometry was used to quantify the T-cell subset populations ($CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^+CD8^+$ and $CD3^+CD4^-CD8^-$) from the PBMCs and monomorphonuclear cells from spleen, the BLN and lungs. Monomorphonuclear cells (1×10^6 cells) were incubated for 20 min on ice with monoclonal antibodies against CD3 (PTT3, IgG1) (Yang et al., 1996), CD4 (74-12-4, IgG2b) (Percovitz et al., 1984) and CD8 (11/295/33, IgG2a) (Saalmuller et al., 1994) (Becton Dickinson, Erembodegem, Belgium). After the wash steps, the cells were incubated with the appropriate isotype-specific FITC rat anti-mouse IgG1 (BD Pharmingen), AlexaFluor®647 goat anti-mouse IgG2a and R-phycoerythrin goat anti-mouse IgG2b (Invitrogen). Mouse IgG1, IgG2 and IgG2b were used as isotype controls. After each incubation step, cells were washed twice with PBS. Finally, the cells were diluted in 400 μ l and analyzed using a FACSCanto flow cytometer (Becton Dickinson Immunocytometry Systems, Erembodegem, Belgium) equipped with two lasers, a 488 nm solid state laser and a 633 nm HeNe laser, and FACSDiva software. For all samples, at least 10,000 cells were counted. All data were corrected for autofluorescence as well as for unspecific bindings using isotype-matched negative controls.

Enumeration of cytokine secreting cells

The numbers of IFN- γ , IL-10 and IL-12 secreting cells in PBMCs and tissue monomorphonuclear cells were determined by use of an ELIspot assay. Ninety-six well microtitre plates were coated with monoclonal antibodies against porcine IFN- γ (Swine IFN- γ Antibody Pair, Invitrogen, Merelbeke, Belgium), IL-10 (IL-10 Swine Antibody Pair, Invitrogen) and porcine IL12/IL23 p40 (Porcine IL-12/IL-23 p40 DuoSet, R&D Systems, MN, USA). After incubating the plates overnight at 4 °C (IFN- γ , IL-10) or at room temperature (IL-12), they were washed with PBS and blocked with PBS + 1% bovine serum albumin (IFN- γ) or RPMI + 10% fetal calf serum (IL-10 and IL-12) for 1 h at 37 °C (IFN- γ , IL-10) or at room temperature (IL-12), followed by a second wash step. Monomorphonuclear cells (5×10^4 /well for IFN- γ , IL-12 and 2×10^4 /well for IL-10) in leukocyte medium alone (no stimulation) or with *M. hyopneumoniae* antigen (10 μ g/ml) were added to the wells, and plates were incubated for 20 h at 37 °C in 5% CO₂. The plates were washed and subsequently, incubated for 1 h at 37 °C with detection antibody diluted in assay buffer (PBS + 0.5% BSA + 0.1% Tween@20 for IL-10 and IFN- γ and PBS + 1% BSA for IL-12). After 3 washes streptavidin-HRP was added. After 1 h incubation at 37 °C, the plates were washed and the spots were developed with insoluble TMB (Sigma, Bornem, Belgium). The plates were scanned and the spots were counted using an Immunospot ELIspot reader (AID GmbH, Strassberg, Germany). Results were presented as the mean of the number of antibody secreting cells stimulated minus no stimulated per 10^5 monomorphonuclear cells.

Secretion of IFN- γ , IL-10, IL-12 and TGF- β in lymphocyte cultures

Lymphocytes collected from the PBMCs, spleen, BLN and lung were seeded in 24-well microtitre plates at a concentration of 10^6 cells/ml in leukocyte medium (no stimulation), leukocyte medium with *M. hyopneumoniae* antigen (10 μ g/ml) or leukocyte medium with phytohaemagglutinin (20 μ g/ml) at 37 °C. Supernatant was collected after 72 h. The quantity of IFN- γ , IL-10 and IL-12 in the supernatant was measured using commercially available kits (Swine IFN- γ Antibody Pair, Invitrogen; IL-10 Swine Antibody Pair, Invitrogen and porcine IL-12/IL-23 p40 DuoSet, R&D Systems). For TGF- β , the TGF- β 1 multispecies (TGF- β 1 Multispecies Antibody Pair, Invitrogen) was used. Samples were processed according to the manufacturer's recommendations. The optical density (OD) at 450 nm was measured and the cytokine levels were quantified by the use of a standard curve.

Detection of cytokines in BAL fluid

Porcine IFN- γ (Swine IFN- γ Antibody Pair, Invitrogen), TNF- α (TNF α Swine Antibody Pair, Invitrogen), IL-1 β (Porcine IL-1 beta/IL-1F2 DuoSet, R&D Systems) and IL-6

(Porcine IL-6 DuoSet, R&D Systems) were measured in BAL fluid using commercially available immunoassay. Samples were processed according to the manufacturer's recommendations. The BAL fluid was tested undiluted. The samples reactions were measured using OD at 450 nm and quantified by the use of a standard curve.

Statistical analyses

Descriptive statistics were performed to check the normality of the variables (T cell populations, cytokines and serum immunoglobulins). In case data did not fulfil the criteria of normality, a non-parametric Kruskal-Wallis ANOVA was used. Results were considered significantly different at $p < 0.05$ (two-sided test). All analyses were performed with SPSS 19.0 for Windows (SPSS inc. Illinois, USA).

Results

Macroscopic lung lesions, nested and qPCR for detection of *M. hyopneumoniae* DNA

In none of the pigs lung lesions typical for *M. hyopneumoniae* infection were observed and BAL from all pigs was negative for the presence of *M. hyopneumoniae* DNA.

Detection of *M. hyopneumoniae* specific serum antibodies

The serological profile of the animals is given in Table 1. Five piglets from the vaccinated and three from the non-vaccinated group were positive at 7 days of age. One non-vaccinated animal remained positive until 36 days. Eleven out of seventeen and eight out of ten vaccinated pigs were positive at 30 and 58 days of age, respectively. From the age of 30 days onwards, the average optical density values significantly differed between both groups.

In the indirect ELISA, OD values for *M. hyopneumoniae*-specific IgM and IgA isotypes did not differ over time or among groups (data not shown). Results for IgG are summarized in Table 2. The OD value decreased in the non-vaccinated group (maternal immunity), whereas in the vaccinated group a decrease was observed until day 36 with a subsequent increase at day 58. The OD value of *M. hyopneumoniae*-specific IgG antibodies in the vaccinated group was higher during the study period, with significant differences at day 7 and 58.

Table 1: Results of serology in non-vaccinated pigs (PBS) and in pigs vaccinated (VACC) against *Mycoplasma hyopneumoniae* at 7, 30, 36 and 58 days of age. Animals were examined for the presence specific serum antibodies to *M. hyopneumoniae* using the IDEIA™ *Mycoplasma hyopneumoniae* ELISA Kit.

	Age of pigs											
	7			30			36			58		
	PBS	VACC	p value	PBS	VACC	p value	PBS	VACC	p value	PBS	VACC	p value
Number of seropositive overall pigs	3/16	5/17	0.400	1/16	11/17	0.001	1/13	13/14	0.000	0/9	8/10	0.001
Number of seropositive necropsied pigs	- ^a	-	-	0/3	2/3	0.400	0/4	4/4	0.029	0/9	8/10	0.001
Average OD value of sera for overall pigs	2.08 ^b (±0.21) ^c	2.05 (±0.24)	0.82	1.26 (±0.10)	0.67 (±0.12)	0.000	1.25 (±0.13)	0.47 (±0.05)	0.000	1.66 (±0.05)	0.84 (±0.19)	0.021

^a No animals were necropsied

^b Optical density at 450 nm

^c ± Standard deviation

Table 2: *Mycoplasma hyopneumoniae*-specific IgG antibody response (mean OD_{670nm} ± standard error of the mean) in the serum at different time-points in the non-vaccinated (PBS) and vaccinated (VACC) pigs. At 7 days of age, pigs were injected intramuscularly with either PBS (n=16) or a commercial *M. hyopneumoniae* inactivated whole cell vaccine (n=17).

Age of the pigs (days)	PBS	VACC	p value
7	0.35 ± 0.07	0.47 ± 0.08	0.015
30	0.26 ± 0.09	0.43 ± 0.14	0.275
36	0.21 ± 0.03	0.26 ± 0.02	0.248
58	0.18 ± 0.01	0.48 ± 0.06	0.001

Determination of T cell populations in different tissues

The T-cell subset populations detected in blood, spleen, BLN and lung tissue are shown in Table 3. There were no statistically significant differences in percentage of lymphocytes subsets between groups at any time evaluated. We did detect a higher percentage of single positive CD8⁺ in the vaccinated group at 30 days. On the other hand, the percentage of CD4⁺ cells was higher in the non-vaccinated group, with a numeric difference in the cells from the BLN ($p=0.05$), with a CD4⁺/CD8⁺ ratio of 3.97 and 2.78 in the non-vaccinated and vaccinated group, respectively, at 30 days. The CD4⁺/CD8⁺ ratio at 30 days of age was higher than 1 in all tissues, and there was a decrease of this ratio in the lung tissue at 58 days of age in both groups. Regarding the CD4⁺CD8⁺ cells, a numeric difference was observed ($p=0.05$) in the BLN at 30 days of age, with a higher percentage of cells in the vaccinated group.

Table 3: Mean percentage \pm standard error of the mean of T-cell subset populations per 10^6 cells from the blood, spleen, bronchial lymph nodes (BLN) and lung, from the non-vaccinated (PBS) and vaccinated (VACC) pigs, assessed using flow cytometry.

Tissue	30 days of age			58 days of age		
	PBS (n=3)	VACC (n=3)	p value	PBS (n=6)	VACC (n=5)	p value
CD4⁺						
Blood	39.6 \pm 5.3	33.9 \pm 7.2	0.513	44.5 \pm 4.8	33.0 \pm 5.7	0.088
Spleen	32.0 \pm 3.9	31.4 \pm 1.5	0.827	31.9 \pm 3.1	32.8 \pm 1.3	0.749
BLN	65.5 \pm 1.1	58.9 \pm 0.4	0.05	64.3 \pm 4.7	61.2 \pm 2.9	0.153
Lung	39.6 \pm 4.5	39.5 \pm 3.6	0.827	22.3 \pm 3.9	21.5 \pm 1.9	0.806
CD8⁺						
Blood	15.7 \pm 2.5	17.3 \pm 4.9	0.827	16.9 \pm 4.5	20.6 \pm 2.3	0.394
Spleen	24.4 \pm 4.4	25.7 \pm 1.1	0.513	32.0 \pm 6.4	26.0 \pm 1.3	0.336
BLN	16.5 \pm 1.8	21.2 \pm 1.3	0.127	17.4 \pm 1.8	16.4 \pm 0.9	0.886
Lung	19.8 \pm 1.9	25.1 \pm 2.5	0.275	34.3 \pm 6.8	37.6 \pm 4.9	0.624
CD4⁺CD8⁺						
Blood	7.4 \pm 0.2	6.9 \pm 0.7	0.658	3.5 \pm 1.8	3.1 \pm 0.8	0.100
Spleen	17.4 \pm 3.9	19.5 \pm 2.2	0.513	10.7 \pm 2.8	14.8 \pm 2.1	0.336
BLN	8.6 \pm 0.6	13.3 \pm 1.9	0.05	12.2 \pm 2.9	17.1 \pm 3.8	0.475
Lung	16.4 \pm 4.5	12.8 \pm 0.1	0.513	9.7 \pm 5.1	6.5 \pm 0.4	1.000
CD4⁻CD8⁻						
Blood	37.3 \pm 2.8	41.9 \pm 9.3	0.827	35.0 \pm 9.5	42.2 \pm 4.4	0.454
Spleen	26.2 \pm 1.7	23.4 \pm 4.7	0.827	25.4 \pm 3.3	26.4 \pm 2.5	0.745
BLN	9.5 \pm 5.0	6.7 \pm 0.9	0.127	6.1 \pm 1.2	5.3 \pm 0.7	0.668
Lung	24.1 \pm 2.6	22.7 \pm 5.7	0.827	33.1 \pm 7.9	34.4 \pm 4.0	0.806

IFN- γ , IL-10 and IL-12 secreting cells

The number of *M. hyopneumoniae*-specific IFN- γ , IL-10 and IL-12 secreting cells is shown in Table 4. A significantly higher number of IL-12 secreting cells was observed in the lung at 58 days of age, and a numeric difference in the spleen at 30 days of age ($p=0.05$) in the vaccinated group. In the BLN, a significantly lower number of IFN- γ secreting cells was observed in the vaccinated than in the non-vaccinated group at 36 days and a significantly higher number of IL-10 secreting cells in the vaccinated group at 58 days.

IFN- γ , IL-10, IL-12 and TGF- β secretion in lymphocyte cultures

IFN- γ , IL-10, IL-12 and TGF- β concentrations in supernatant of lymphocytes isolated from blood, spleen, BLN and lung is shown in Table 5. Significantly higher amounts of IL-12 were obtained from BLN and lungs in the vaccinated group at 58 days of age. Not significant differences for IFN- γ , IL-10 and TGF- β , were observed. However, higher amounts of IL-10, statistically not significant, were detected in the BLN from the vaccinated group at all time points evaluated.

***M. hyopneumoniae*-specific IgG, IgM and IgA in BAL fluid**

The three isotypes were detected in BAL fluid of vaccinated animals. *M. hyopneumoniae* specific IgM antibodies were detected at day 30 in all animals necropsied from the vaccinated group. For IgG, three out of 10 animals were positive at day 58 in the vaccinated group. In contrast, specific anti-*M. hyopneumoniae* IgA antibodies were detected at each time point in the vaccinated animals, with two out of three positive animals at day 30, four out four at day 36 and six out of ten animals at day 58. For isotypes and time points specified as above, a statistically significant difference ($p<0.05$) was observed between control and vaccinated animals when analysing OD values.

IFN- γ , TNF- α , IL-1 β and IL-6 concentrations in BAL fluid

For IFN- γ at 30 days of age a significantly higher concentration was detected ($p=0.046$) in BAL fluid of the vaccinated group (Fig. 1a). For TNF α , a significantly higher concentration was measured at day 36 ($p=0.040$) in the vaccinated group, but the overall TNF- α concentration was quite similar between both groups over the study period (Fig. 1b). IL-1 β and IL-6 (Fig. 1c and 1d) were detected at low levels in the vaccinated group, with a significant difference at day 36 for IL-1 β ($p=0.043$).

Table 4: Mean number of IFN- γ , IL-10 and IL-12 secreting cells per 10^5 monomorphonuclear cells \pm standard error of the mean in blood, spleen, bronchial lymph nodes (BLN) and lung from the non-vaccinated (PBS) and vaccinated (VACC) pigs at different time points measured by ELISpot.

	30 days of age			36 days of age			58 days of age		
	PBS (n=3)	VACC (n=3)	p value	PBS (n=4)	VACC (n=4)	p value	PBS (n=7)	VACC (n=8)	p value
IFN-γ									
Blood	0.6 \pm 0.6	5.0 \pm 3.7	0.178	3.0 \pm 2.1	1.3 \pm 0.6	0.881	7.9 \pm 3.7	12.3 \pm 4.6	0.409
Spleen	0.0 \pm 0.0	7.6 \pm 3.9	0.246	11.0 \pm 11.0	17.3 \pm 5.5	0.237	8.7 \pm 5.6	15.0 \pm 9.3	0.676
BLN	0.0 \pm 0.0	0.3 \pm 0.1	0.246	6.5 \pm 4.3	0.0 \pm 0.0	0.047	3.2 \pm 1.8	9.6 \pm 4.8	0.127
Lung	1.2 \pm 0.6	0.5 \pm 0.2	0.275	5.7 \pm 2.1	0.7 \pm 0.5	0.137	0.1 \pm 0.1	2.1 \pm 1.1	0.055
IL-10									
Blood	217.3 \pm 17.9	252.3 \pm 50.6	0.513	250.0 \pm 170.9	106.3 \pm 40.1	0.885	320.4 \pm 105.7	390.6 \pm 83.7	0.353
Spleen	129.7 \pm 50.6	95.3 \pm 24.3	0.513	136.3 \pm 49.7	118.8 \pm 40.9	0.885	248.9 \pm 91.1	161.6 \pm 53.7	0.441
BLN	6.7 \pm 6.7	3.0 \pm 2.1	0.817	6.3 \pm 2.6	2.5 \pm 1.4	0.225	0.4 \pm 1.9	10.0 \pm 6.0	0.031
Lung	39.7 \pm 20.0	60.3 \pm 19.9	0.275	123.8 \pm 49.9	191.3 \pm 58.3	0.248	91.1 \pm 26.3	173.8 \pm 43.8	0.132
IL-12									
Blood	0.4 \pm 0.2	0.3 \pm 0.18	0.637	0.3 \pm 0.3	0.0 \pm 0.0	0.317	12.9 \pm 7.0	11.6 \pm 3.9	0.723
Spleen	0.3 \pm 0.2	5.3 \pm 2.64	0.050	9.0 \pm 2.8	12.3 \pm 4.9	0.663	34.6 \pm 14.8	23.9 \pm 8.9	0.350
BLN	0.0 \pm 0.0	0.13 \pm 0.13	0.317	1.0 \pm 0.6	0.8 \pm 0.3	0.760	7.3 \pm 3.	7.0 \pm 2.7	0.770
Lung	0.1 \pm 0.1	0.1 \pm 0.03	0.346	1.8 \pm 1.2	1.5 \pm 1.5	0.741	2.3 \pm 1.6	8.0 \pm 2.6	0.036

Table 5: Mean IFN- γ , IL-10, IL-12 and TGF- β concentration \pm SEM (pg/ml) in lymphocyte cultures from blood, spleen, bronchial lymph nodes (BLN) and lungs in the non-vaccinated (PBS) and vaccinated (VACC) pigs at different time points measured by ELISA.

	Day 30			Day36			Day 58		
	PBS (n=3)	VACC (n=3)	p value	PBS (n=4)	VACC (n=4)	p value	PBS (n=7)	VACC (n=8)	p value
IFN-γ									
Blood	81.1 \pm 5.4	72.9 \pm 20.4	0.513	141.2 \pm 36.0	103.5 \pm 32.1	0.149	101.9 \pm 7.1	103.2 \pm 4.9	0.817
Spleen	65.3 \pm 4.8	102.8 \pm 16.6	0.127	151.0 \pm 54.1	142.3 \pm 48.4	0.100	102.1 \pm 8.6	105.8 \pm 8.1	0.817
BLN	142.6 \pm 9.2	126.4 \pm 6.4	0.275	135.6 \pm 39.6	130.9 \pm 39.3	0.564	100.3 \pm 6.8	104.7 \pm 7.4	0.817
Lung	77.1 \pm 13.5	73.7 \pm 11.6	0.827	154.0 \pm 48.4	111.3 \pm 43.4	0.248	93.9 \pm 5.2	105.9 \pm 6.2	0.083
IL-10									
Blood	1196.3 \pm 75.1	1463.8 \pm 300.3	0.513	1398.0 \pm 122.9	1346.0 \pm 23.3	0.773	1455.1 \pm 188.3	133.1 \pm 137.9	0.817
Spleen	2002.3 \pm 837.6	2957.7 \pm 295.8	0.275	1371.3 \pm 215.8	1358.8 \pm 63.2	0.773	1475.3 \pm 203.4	1669.1 \pm 119.2	0.563
BLN	499.0 \pm 85.7	902.3 \pm 247.2	0.127	527.8 \pm 117.7	547.3 \pm 119.1	0.564	489.4 \pm 123.4	617.1 \pm 94.0	0.298
Lung	900.0 \pm 257.0	960.3 \pm 161.5	0.827	1238.8 \pm 219.0	1042.0 \pm 112.2	0.248	904.3 \pm 124.9	944.25 \pm 115.10	0.728
IL-12									
Blood	102.3 \pm 51.9	55.3 \pm 35.3	0.376	170.3 \pm 116.4	278.0 \pm 180.5	0.758	232.3 \pm 93.2	178.0 \pm 75.6	0.550
Spleen	1933.7 \pm 641.9	1807.7 \pm 788.1	0.827	1345.8 \pm 212.7	3367.5 \pm 2015.0	0.564	1539.1 \pm 422.5	2532.4 \pm 338.8	0.132
BLN	0.0	801.3 \pm 737.8	0.121	590.0 \pm 276.8	452.0 \pm 285.2	0.663	472.7 \pm 230.1	1196.0 \pm 210.7	0.028
Lung	268.7 \pm 77.4	541.30 \pm 185.0	0.275	155.0 \pm 54.9	337.8 \pm 129.3	0.191	255.9 \pm 83.5	702.4 \pm 119.9	0.018
TGF-β									
Blood	308.0 \pm 252.2	405.7 \pm 225.0	0.275	266.8 \pm 38.9	481.0 \pm 150.4	0.149	709.0 \pm 102.9	447.1 \pm 119.4	0.105
Spleen	239.3 \pm 125.6	152.7 \pm 55.4	0.513	263.0 \pm 121.3	56.0 \pm 33.7	0.081	252.9 \pm 142.7	304.0 \pm 169.9	0.896
BLN	63.3 \pm 32.2	158.0 \pm 51.0	0.275	296.8 \pm 85.2	429.0 \pm 130.7	0.386	737.0 \pm 152.5	416.0 \pm 123.6	0.064
Lung	343.0 \pm 214.1	465.7 \pm 136.0	0.513	252.3 \pm 100.5	259.3 \pm 188.7	0.564	364.0 \pm 194.0	382.9 \pm 151.5	0.858

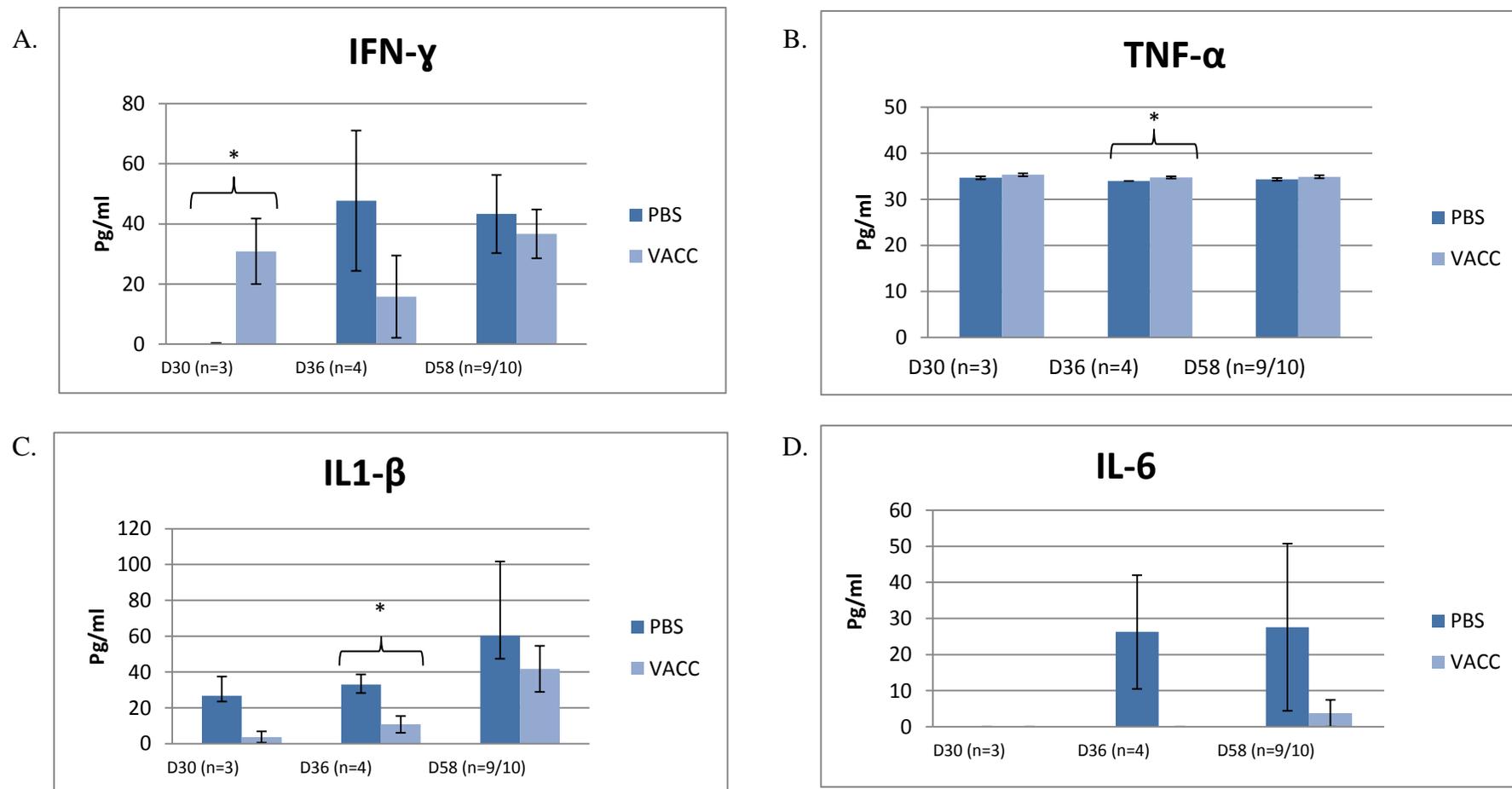


Figure 1: Mean (and SE) of IFN- γ (A), TNF- α (B), IL-1 β (C) and IL-6 (D) secretion in BAL fluid measured by ELISA (OD_{450nm}) from the non-vaccinated (PBS) and vaccinated (VACC) pigs, at the different time points. Pigs were vaccinated at 7 days of age with a commercial *M. hyopneumoniae* inactivated whole cell vaccine.

Discussion

As expected, vaccination with the current bacterin induced the production of serum antibodies against *M. hyopneumoniae*. Almost 70% of the pigs were positive within three weeks after vaccination and 80% of the animals were positive at the end of the study. These results are in agreement with other studies (Sibila et al., 2007; Villarreal et al., 2011) where one-shot vaccination at 7 days of age induced seroconversion by the 25th day of age. However, previous challenge experiments showed that the concentration of serum antibodies is not directly related to protection against *M. hyopneumoniae* infections (Djordjevic et al., 1997; Thacker et al., 1998).

Studies carried out in vaccinated (Borghetti et al., 2006; Kick et al., 2011) and non-vaccinated pigs (Stepanova et al., 2007) showed that different T cell subsets may be present in the peripheral blood and secondary lymphoid tissues. Our results were partially consistent with these reports, with an increase of the percentage of CD8⁺ T cells with increasing age in both groups and in all tissues (except in the BLN of vaccinated group). Previous reports also showed a decrease of CD4⁺ and an increase in the percentage of CD4⁺CD8⁺ T cells with increasing age (Borghetti et al., 2006; Kick et al., 2011). In our studies, this decreased percentage of CD4⁺ cells was seen in lung tissue and an increased percentage of CD4⁺CD8⁺ T cells was observed in the BLN.

In most tissues the percentage of CD8⁺ T cells was highest in the vaccinated group and at day 58, the percentage of CD8⁺ cells prevailed over CD4⁺ cells in the lung tissue. CD8⁺ T cells may modulate the inflammatory response through the production of IFN- γ and other cytokines, minimizing damage due to the CD4⁺ T (Th) cell mediated inflammatory responses (Jone and Simecka, 2003). This might be one of the reasons why vaccinated animals develop less severe clinical symptoms and lung lesions after challenge with *M. hyopneumoniae* (Meyns et al., 2006; Villarreal et al., 2011).

At 58 days of age, a significantly higher number of IL-10 secreting cells was detected in the BLN tissue of the vaccinated animals. Since IL-10 has an anti-proliferative effect, that can prevent the pathological effects of the inflammatory cytokines (Conti et al., 2003), this may also play a role in the reduction of lung tissue damage after infection of vaccinated animals which has been described in several studies (Meyns et al., 2006; Villarreal et al., 2011). Induction of IL-10 secretion by vaccination may also result in a lower influx of macrophages in the bronchoalveolar lymphoid tissue in vaccinated pigs compared to non-vaccinated animals, after experimental infection with *M. hyopneumoniae*, as described by Vranckx et al. (2012).

To evaluate the specific *M. hyopneumoniae* cell-mediated immune response induced by vaccination, ELISA and ELISpot were performed to quantify IFN- γ , IL-10 and IL-12 cytokines and cytokines producing cells. Vaccinated pigs showed significantly higher amounts of IL-12 in BLN and lung tissues. Production of IL-12 by macrophages and B cells has been demonstrated to be important in the induction of a Th1 immune response (Yang et al., 2004). Furthermore, this cytokine stimulates IFN- γ production and enhances CD8⁺ cytotoxicity, both contributing to maintaining the cell mediated immunity (Park and Scott, 2001). Indeed, at day 58, the number of IFN- γ secreting cells was numerically higher ($p=0.055$) in the lungs, and significantly higher in the BAL fluid of the vaccinated group at day 30, indicating that the vaccine also induced a local cellular immune response.

The highest concentrations of IL-1 β and IL-6 were detected in the BAL fluid of the non-vaccinated group, with a significantly higher value at day 36 for IL-1 β . This may suggest a regulatory effect of the vaccine in the release of these cytokines. Increased levels of IL1 and IL6 were indeed observed in BAL fluid of *M. hyopneumoniae* infected pigs (Choi et al., 2006; Thacker et al., 2000) and excessive levels of these cytokines have been associated with tissue damage and even death of the host (Asai et al., 1993; Okada et al., 2000).

In the present study, vaccination was able to stimulate IgG, IgM and IgA specific *M. hyopneumoniae* antibodies in the BAL fluid. IgA specific antibodies were detected at all time points investigated. Specific antibodies in the BAL fluid may play an important role in protection against *M. hyopneumoniae* infections (Thacker et al., 2000). *M. hyopneumoniae* is a mucosal pathogen that adheres to the ciliated epithelial cells of the respiratory tract. Local IgA secretion prevents the adhesion of mycoplasmas to the ciliated epithelium, and IgG enhances their phagocytosis by alveolar macrophages (Sheldrake et al., 1990).

In conclusion, the present study documented that the vaccine induced both local and systemic immune responses involving both specific antibodies and cellular immunity. Further studies are necessary to determine the role of these components of the host immune response in protection induced by the vaccine.

Conflict of interest statement

The authors state they have no conflict of interest. The study was funded by Pfizer Animal Health, manufacturer of the vaccine used in this study. Wuyts N. is employee of Pfizer Animal Health.

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CHAPTER 3.2:

Immune response induced by a recombinant chimeric protein containing *Mycoplasma hyopneumoniae* antigens fused to the B subunit of Escherichia coli heat labile enterotoxin LTB

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Abstract

Multi-antigen chimera with three antigens of *Mycoplasma hyopneumoniae* (R1, P42 and NrdF) and the mucosal adjuvant *Escherichia coli* heat labile enterotoxin B subunit (LTB) was constructed, and its antigenic and immunogenic properties were evaluated in mice and pigs. In addition, we compared the effect of the fusion and co-administration of these proteins. Antibodies against each subunit recognized the chimeric protein. Intranasal and intramuscular immunization of mice with the chimeric protein significantly increased the levels of mice IgG and IgA, in serum and tracheobronchial lavages, respectively. Swine immunized with the chimeric protein developed an immune response against all *M. hyopneumoniae* antigens present in the fusion with statistically significant difference ($p < 0.05$). The adjuvant rLTB stimulated the immune response in both, fused and co-administered antigens, with better results with the chimeric protein. This multi-antigen is a promising vaccine candidate that may help to control *M. hyopneumoniae* infection.

Keywords: Fused protein, swine enzootic pneumonia, adjuvant, bacterial enterotoxins

Introduction

Mycoplasma hyopneumoniae, the primary pathogen of enzootic pneumonia (EP), occurs worldwide and causes major economic losses to the pig industry (Thacker and Minion, 2010). Vaccination combined with hygiene and management procedures is the most effective way to control the disease (Maes et al., 2008). The current commercial vaccines consist of whole-cell adjuvanted formulations, and provide only partial protection. They do not prevent colonization of *M. hyopneumoniae* in the respiratory tract, do not significantly reduce transmission, and are costly to produce (Haesebrouck et al., 2004).

Major efforts have been made towards the development of improved vaccines using diverse strategies, mainly through the use of subunit vaccines, rather than those based on whole bacterial cells. Subunit vaccines are considerably safer, more specific, with less adverse reactions and with the possibility of large scale production of recombinant proteins by biotechnological process (Soria-Guerra et al., 2011). They can also be easily manipulated and administered to the animal through various routes such as oral, nasal, intramuscular, or subcutaneous. In addition, potentially multiple antigens can be delivered at the same time in a single dose of immunization as chimeric proteins (Liljeqvist and Stahl, 1999). Chimeric proteins carrying epitopes from different pathogens, linkers, or adjuvant sequences offer an increased immunogenicity of recombinant antigens, and can also elicit a broad cellular and/or humoral immune response (Berzofsky et al., 2001).

Considering that genetically engineered vaccines composed of a single recombinant antigen are poorly immunogenic, it is important to increase their immunogenicity by combining with appropriate adjuvants (Soria-Guerra et al., 2011). One strategy has been to employ bacterial enterotoxins such as cholera toxin (CT) from *Vibrio cholera* or the related subunit B of heat-labile enterotoxin of *Escherichia coli* (LTB). These have been shown to be highly immunogenic, demonstrate potent immunomodulatory activity with a range of antigens either fused or co-administered to them, in different animal models, enhance immunogenicity as well as to confer protective efficacy (Conceição et al., 2006; da Hora et al., 2011; Grassmann et al., 2012; Qiao et al., 2009; Zhou et al., 2009). They are considered as potent mucosal and parenteral adjuvants (Cox et al., 2006; da Hora et al., 2011; Simmons et al., 2001), which have been demonstrated to enhance mucosal immunoglobulin A (IgA) as well as systemic antibody responses.

The reverse vaccinology approach revealed that several *M. hyopneumoniae* antigens might be potential candidates to be included in subunit vaccines (Fagan et al., 2001; Marchioro et al., 2012; Ogawa et al., 2010; Okamba et al., 2010; Okamba et al., 2007;

Shimoji et al., 2003; Simionatto et al., 2012). Some of these antigens include the P97 adhesin and its C-terminal region (P97R1) which plays a role in adherence of the pathogen to the respiratory tract of the host, the ribonucleotide reductase (NrdF) and the heat shock protein P42. P97 (Shimoji et al., 2003), P97R1 (Ogawa et al., 2010; Okamba et al., 2010) and NrdF (Fagan et al., 2001) have been investigated as single antigens in different vaccine formulations. Some of these formulations (P97 and NrdF) were able to induce specific immune responses in pigs (Fagan et al., 2001; Shimoji et al., 2003), but provided only limited protection against pulmonary lesions caused by *M. hyopneumoniae* infection. However, improved strategies for efficient antigen presentation and immune responses are still needed to increase protective immunity against infection.

The aims of the present study were (i) to produce a recombinant chimeric protein composed of the P97R1, P42 and NrdF antigens of *M. hyopneumoniae* fused to LTB, (ii) to evaluate the capacity of this chimeric protein to induce a specific immune response in mice and pigs and (iii) to compare the effect of the fusion and co-administration of these proteins as well as the adjuvant effect of LTB.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli TOP 10 (Invitrogen) and BL21 (DE3) RIL (Stratagene) were grown in Luria Bertani (LB) medium supplemented with ampicillin ($100 \mu\text{g}\cdot\text{ml}^{-1}$), as required, and incubated in shaker at 37°C and 200 rpm.

Plasmids, DNA procedures and electrotransformation

The plasmids encoding the gene *p42*, *nrdF* and *p97(r1)* of *M. hyopneumoniae* strain 7448 (GenBank accession number [NC007332](#)) and the *ltb* of *E. coli* were cloned in our laboratory as previously described (Conceição et al., 2006; Simionatto et al., 2010). A synthetic chimeric gene encoding the *E. coli ltb*, the R1 repeat region of *p97 (r1)*, the C-terminal portion of heat shock protein gene *p42* and the ribonucleotide reductase *nrdF* of *M. hyopneumoniae* was obtained from Epoch Life Science (Texas, US). The sequence encoding the chimeric gene was provided with restriction sites necessary for further cloning step in *E. coli* expression vector - pAE (Ramos et al., 2004). The chimeric gene was excised from the pUC18 plasmid provided by Epoch Life Science using the *Bam*HI and *Kpn*I restriction enzymes and cloned into corresponding sites of pAE *E. coli* expression vector. *E. coli* Top 10 competent cells were transformed by electroporation with ligation products as described by Sambrook and Russel (2001). Recombinant clones were selected and plasmid DNA was

extracted and characterized by digestion with restriction enzymes and DNA sequencing using a MegaBACE 500 DNA sequencer and the Dynamic ET-terminator technology (GEHealthcare).

Expression, solubility testing and purification of recombinant proteins

The recombinant vectors pAE/*ltb*, pAE/*p42*, pAE/*nrdF*, pET/*r1*, and pAE/*ltbr1p42nrdF* were used to transform *E. coli* BL21 (DE3) Ril. The 6X His-tagged recombinant LTB (rLTB), recombinant P42 (rP42), recombinant NrdF (rNrdF), recombinant R1 (rR1) and recombinant chimeric protein (rLTBR1P42NrdF) were expressed and purified by affinity chromatography as previously described (Marchioro et al., 2012; Simionatto et al., 2010). Fractions containing recombinant proteins were identified by SDS-PAGE and quantified by BCA™ Protein Assay (Pierce) following the manufacturer's instructions.

Characterization of the recombinant chimeric protein by Western blot

Purified recombinant proteins rLTB, rR1, rP42, rNrdF and chimeric protein rLTBR1P42NrdF were applied to 12% SDS-PAGE and electro-transferred on a nitrocellulose membrane for characterization by Western blot analysis as described elsewhere (Simionatto et al., 2010). The antibodies and dilutions used were: monoclonal anti-R1 (1:4000), polyclonal anti-P42 (1:100), polyclonal anti-NrdF (1:100) or polyclonal anti-LTB (1:100) and a HRP-conjugate goat anti-mouse (Sigma Aldrich) diluted 1:6000.

The ability of rLTBR1P42NrdF to bind to GM1-ganglioside was determined by an enzyme-linked immunosorbent assay (ELISA) as previously described (Grassmann et al., 2012), using the following antibodies and dilutions: monoclonal anti-R1 (1:4000), polyclonal anti-P42 (1:100), polyclonal anti-NrdF (1:100) or rabbit IgG anti-cholera toxin (1:6000) followed by a goat IgG anti-mouse or anti-rabbit IgG peroxidase conjugated diluted 1:6000. Wells with GM1 but without proteins and wells without GM1 but with proteins were used as controls.

Immunogenicity in mice

Animal experiments were carried out according to the guidelines of the Ethical Committee in Animal Experimentation of the Federal University of Pelotas. Female BALB/c mice aged 7 to 8 weeks were used in the experiment. Eight groups of mice with eight animals each were inoculated intramuscularly (IM) and or intranasally (IN) on days 0, 15 and 30 with 20 µg of recombinant proteins as follow: (1) rLTB IN; (2) rLTB IM; (3) rLTBR1P42NrdF (chimeric protein) IN; (4) rLTBR1P42NrdF IM; (5) rR1+rP42+rNrdF+ rLTB (co-administered proteins with adjuvant) IN; (6) rR1+rP42+rNrdF+ rLTB IM; (7) rR1+rP42+rNrdF (co-administered proteins without adjuvant) IN; (8) rR1+rP42+rNrdF IM.

For groups 5, 6, 7 and 8, the doses of each protein were calculated in order to have the equivalent amount of each protein as contained in the chimeric protein. Blood samples were collected from the retro-orbital sinus at 0, 15, 30 and 45 days post inoculation (DPI). The sera were processed and stored at -20 °C until tested. On day 45, the animals were euthanized and in four mice of each group, tracheobronchial lavages were collected. Tracheobronchial lavages were carried out by flushing three times with a total volume of 0.5 ml of PBS containing 0.5% bovine serum albumin (BSA) and a protease inhibitor PMSF (inhibitor phenylmethylsulfonyl fluoride; Sigma) 0.1 mM and stored at -20 °C.

To assess the immunogenicity of chimeric protein, sera from mice (diluted 1:100) before and 30 days after the first immunization with this protein were confronted with 2 µg of extract of different *M. hyopneumoniae* strains (J, 7448 and USP strain) by Western blot as described above.

Immunogenicity in pigs

Three *M. hyopneumoniae* free piglets were used to check the immunogenicity of the chimeric protein. Animals were inoculated intramuscularly with three doses of 300 µg of the chimeric protein in 10 days interval. Serum samples were collected and systemic IgG was measured by indirect ELISA.

Antibody assays

The specificity of the antibodies elicited by immunization with the chimeric protein and recombinant proteins co-administrated in mice and pigs was determined by an indirect ELISA. Microtiter plates were coated with 100 µL of each recombinant protein at concentrations ranging from 10 to 100 ng/well in 0.1 M sodium carbonate buffer (pH 9.6), at 4 °C overnight. The plates were washed three times with PBST and incubated with 200 µL of 5% blocking buffer at 37 °C for 2 h. After washing with PBST, wells were incubated with mice or pig sera (diluted 1:100 in blocking buffer) and or tracheobronchial lavages (undiluted), at 37 °C for 1 h. After washing three times with PBST, goat anti-mouse or anti-pig IgG peroxidase conjugated (Sigma Aldrich) and or anti-mouse IgA peroxidase conjugate (Sigma Aldrich) diluted 1:6000 was added and the plate was incubated at 37 °C for 1 h. The reactions were developed with *o*-phenylenediaminedihydrochloride (Sigma) and hydrogen peroxide, after PBST washes. The color reaction was allowed to develop for 15 min before being stopped with 50 µl of sulfuric acid 1 N. Absorbance was determined at 492 nm with a microplate reader (Multiskan MCC/340, Titertek Instruments). Mean values were calculated from serum samples assayed in triplicate. The IgG isotype profiles were also determined, anti-mouse IgG was substituted by antibodies supplied by IsoQuick™ Kit for Mouse Monoclonal

Isotyping (Sigma Aldrich) according to the manufacturer's instructions using mice serum samples obtained at day 0 and 45.

Statistical Analysis

All data were expressed as mean \pm standard deviation. Experimental values of IgG antibody responses were analyzed by analysis of variance (ANOVA), data were log-transformed to conform to the normality and variance requirements of parametric testing. If the p value from the ANOVA was less than 0.05, pairwise comparisons of the different treatment groups were performed by Scheffé test at a rejection level of a p -value < 0.05 . The non-parametric Kruskal-Wallis test was used for comparison of IgA antibody responses. Statistical analyses were performed using SPSS Statistical Software 19.

Results

Recombinant proteins expression and antigenicity

Protein electrophoresis showed bands with apparent molecular masses of each recombinant protein. rLTB, rP42, rNrdF and rLTBR1P42NrdF were expressed in inclusion bodies and required the addition of the denaturing agent *N*-lauroyl-sarcosine for purification, while rR1 was expressed and purified as a soluble protein. The antigenicity of the rLTBR1P42NrdF chimeric protein was confirmed by Western blot using anti-R1, anti-P42, anti-NrdF and anti-LTB antibodies. The antibodies recognized all the proteins contained in the chimeric protein.

The GM1-ELISA with recombinant proteins revealed GM1-binding affinity of rLTB and rLTBR1P42NrdF, showing that the fusion did not impair the binding affinity of LTB (data not shown).

Immunogenicity in mice

The capacity of the chimeric protein and the co-administrated proteins to induce serum IgG antibodies in mice was evaluated by ELISA. All formulations stimulated detectable antibodies against each protein, showing that the chimeric and co-administered recombinant proteins were immunogenic for mice. Besides, antibodies induced by the immunization with the chimeric protein were able to react with all *M. hyopneumoniae* strains tested by Western blot, with a weaker reaction with the non-pathogenic J strain (data not shown).

In the groups immunized IM, the IgG level was higher when compared with IN route, as well as the response to the protein rP42 was higher when compared with the other two proteins (Fig. 1A, 1B, 1C). The anti-rP42 and anti-rR1 antibodies were higher in sera from mice vaccinated with the chimeric protein, with significant differences at 30 and 45 DPI

($p < 0.05$) (Fig. 1A, 1B and 1D). Anti-rNrdF antibodies in the serum of animals inoculated IM with the chimeric protein had the lowest reactions compared with the other two proteins, with highest levels of antibodies in the group inoculated with the proteins co-administrated with LTB (Fig. 1C). Nevertheless, no difference between the chimeric protein group and the group that received the proteins co-administered with adjuvant LTB was found at 45 DPI against all the three antigens. Intranasal immunization did not show significant differences in antibody production against rR1 and rNrdF proteins.

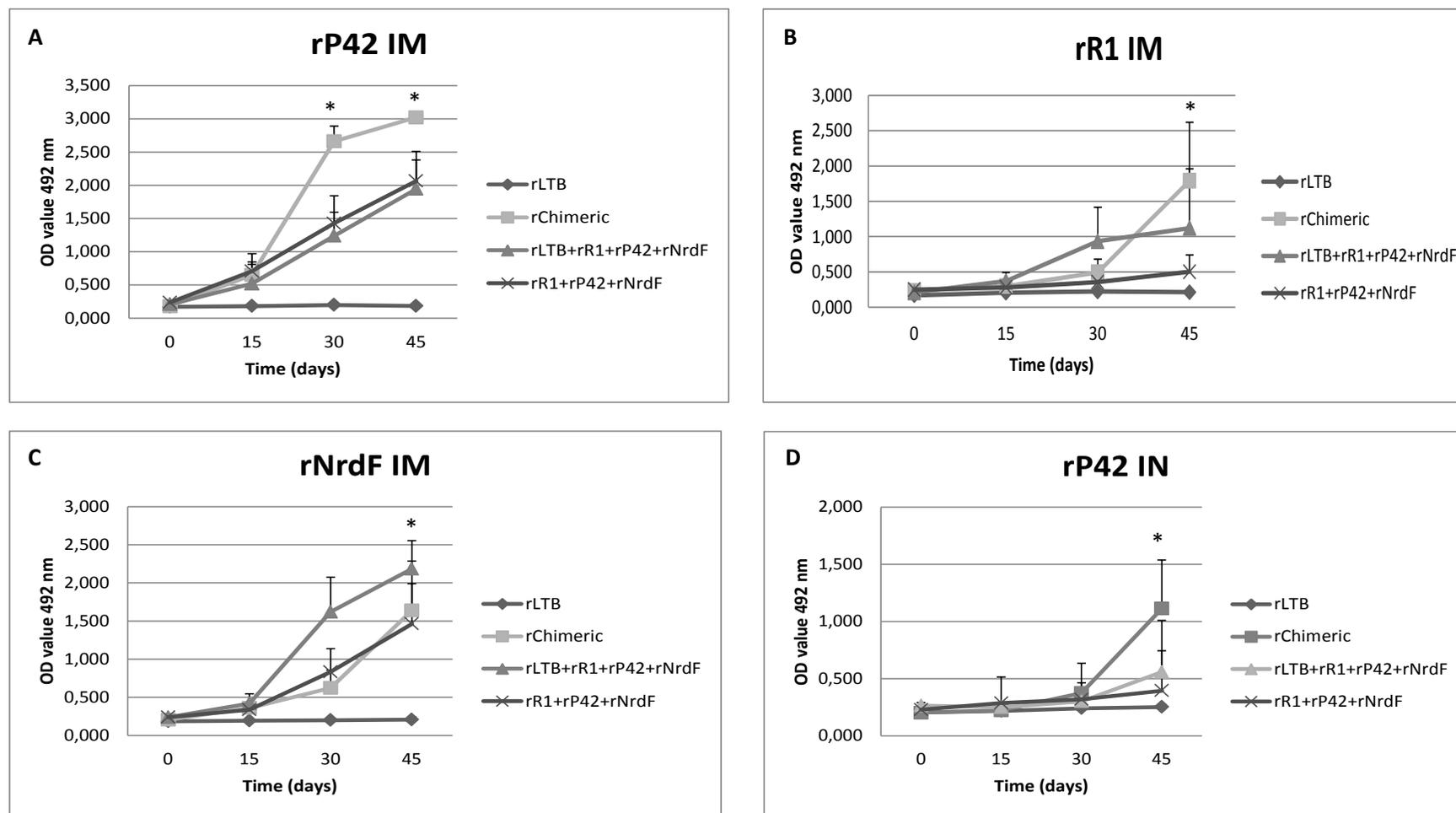


Figure 1. Absorbance at 492 nm of serum of mice immunized intramuscularly (A, B and C) or intranasally (D) with rLTB, chimeric protein, rLTB+rR1+rP42+rNrdF or with rR1+rP42+rNrdF at 0, 15 and 30 days post inoculation (DPI). On days 0, 15, 30 and 45, sera of mice were obtained and the levels of anti-rP42, rR1 and rNrdF were determined by indirect ELISA. Microtiter plates were coated with recombinant proteins and incubated with sera from immunized mice diluted 1:100. Anti-mouse IgG peroxidase conjugated (1:6000) was added as secondary antibody. Data represent the mean and standard deviation OD₄₉₂ (n=8) of mice serum. All reactions were performed in triplicate. **p*<0.05 compared with the mice treated with rLTB, rLTB+rR1+rP42+rNrdF or with rR1+rP42+rNrdF.

Mucosal IgA production in tracheobronchial lavages from different groups at 45 DPI was also tested (Fig. 2). No differences between IM or IN administration were detected and significant differences were only found in the IgA antibodies against the rP42. Levels of IgA anti-rP42 antibodies in groups vaccinated with chimeric protein by both routes of immunization showed the highest levels, with statistically difference from the control group (LTB by IM or IN) and from the group that received co-administered protein without adjuvant by IM ($p<0.05$). The anti-rR1 and anti-rNrdF IgA antibodies did not show significant differences between the groups evaluated.

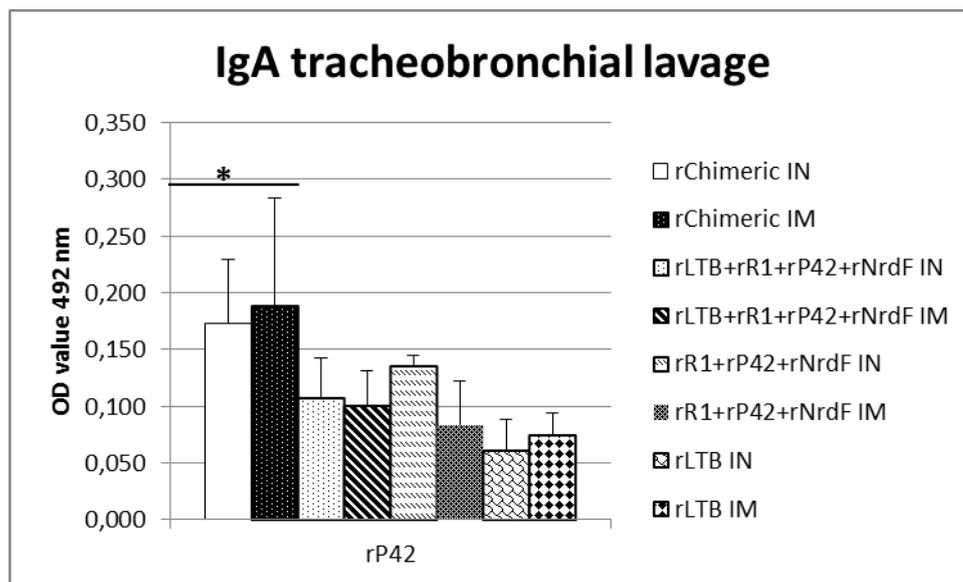


Figure 2. Absorbance at 492 nm of tracheobronchial lavages from mice immunized intramuscularly (IM) or intranasally (IN) with rLTB, chimeric protein, rLTB+rR1+rP42+rNrdF or with rR1+rP42+rNrdF at 0, 15 and 30 DPI. On day 45 the animals were euthanized, tracheobronchial lavages collected and the levels of anti-rP42 were determined by indirect ELISA. Microtiter plates were coated with recombinant protein and incubated with undiluted tracheobronchial lavage. Anti-mouse IgA peroxidase conjugated (1:6000) was added as secondary antibody. Data represent the mean and standard deviation OD₄₉₂ (n=4) of mice tracheobronchial lavage. All reactions were performed in triplicate. * $p<0.05$ compared with the mice treated with rLTB (IM and IN), rLTB+rR1+rP42+rNrdF (IN) or with rR1+rP42+rNrdF (IM).

Analysis of IgG isotypes showed that all the groups induced predominantly IgG1. By IM, the group inoculated with the chimeric protein showed the highest seroconversion for both isotypes against all the proteins, with significant differences to all the groups ($p<0.05$). Although the induction of IgG1 was higher (Fig. 3A), an induction of IgG2a (Fig. 3B) was observed. In the groups immunized IN, highest and significant seroconversion against the protein rP42 was observed in the group immunized with chimeric protein, that showed significant differences within all the groups ($p<0.05$) for both isotypes (Figs. 3C and D).

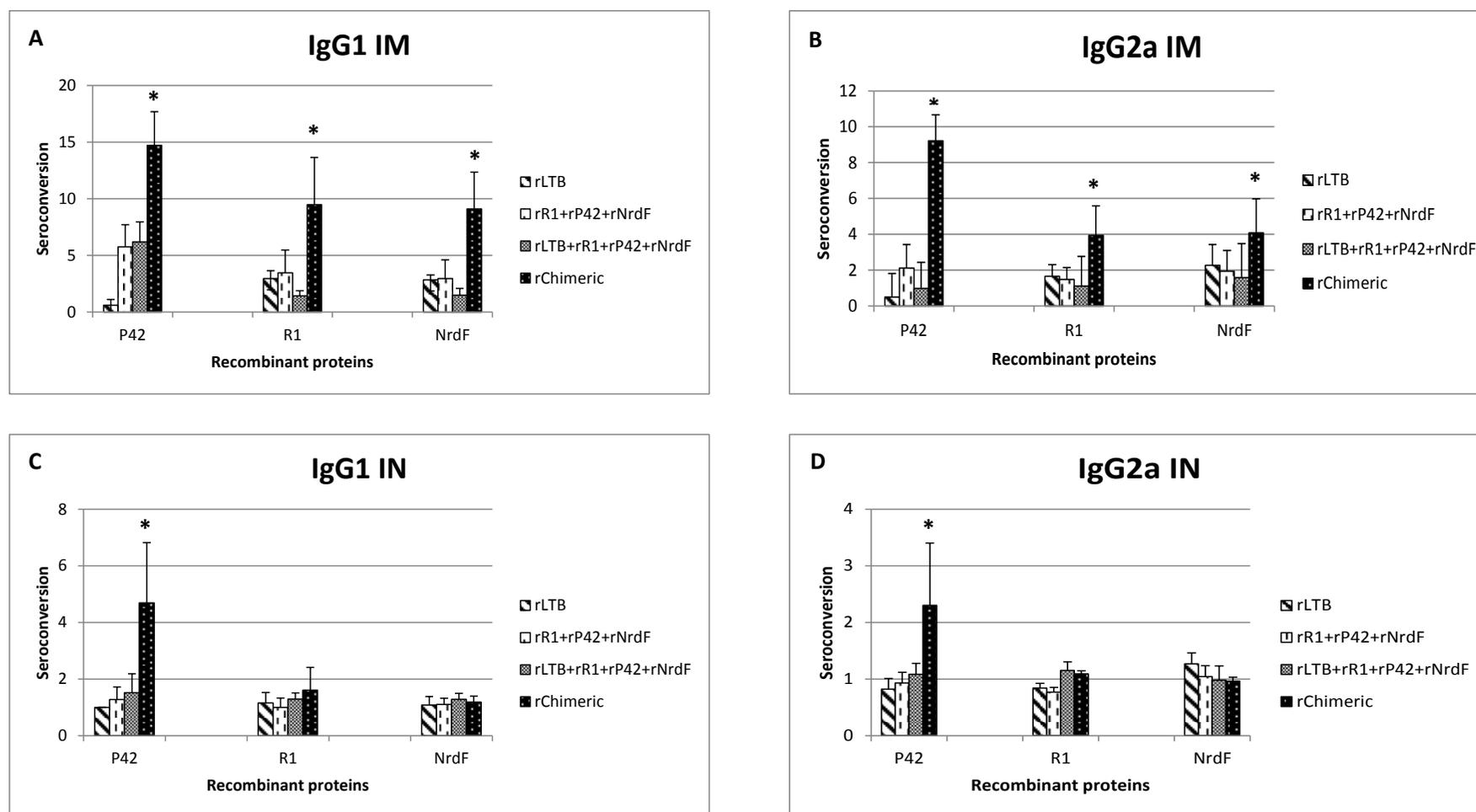


Figure 3. Recombinant proteins-specific IgG1 and IgG2a antibodies determined by ELISA. Microtiter plates were coated with recombinant proteins (rR1, rP42 and rNrdF). Recombinant proteins were incubated with sera (diluted 1:100) from immunized mice intramuscular (A and B) and intranasal (C and D). Goat anti-mouse IgG1 and IgG2a supplied by IsoQuick™ Kit for Mouse Monoclonal Isotyping (Sigma) were used as secondary antibody. Data represent the mean OD₄₉₂ (n=8) of mice serum collected at 45 DPI divided by mean OD₄₉₂ of mice serum collected at day 0. All reactions were performed in triplicate. * $p < 0.05$ compared with the mice treated with rLTB, rLTB+R1+P42+NrdF and with rR1+P42+NrdF.

Immunogenicity in pigs

The humoral immune response induced by the chimeric protein administered IM was evaluated in pigs by ELISA (Fig. 4). The fusion induced a humoral immune response. Similarly to what was observed in mice, the antibodies anti-rP42 showed higher levels when compared to the other proteins, although antibodies anti-NrdF and anti-R1 were also detected with significant difference between the day 0 (pre-immune) and 30 DPI.

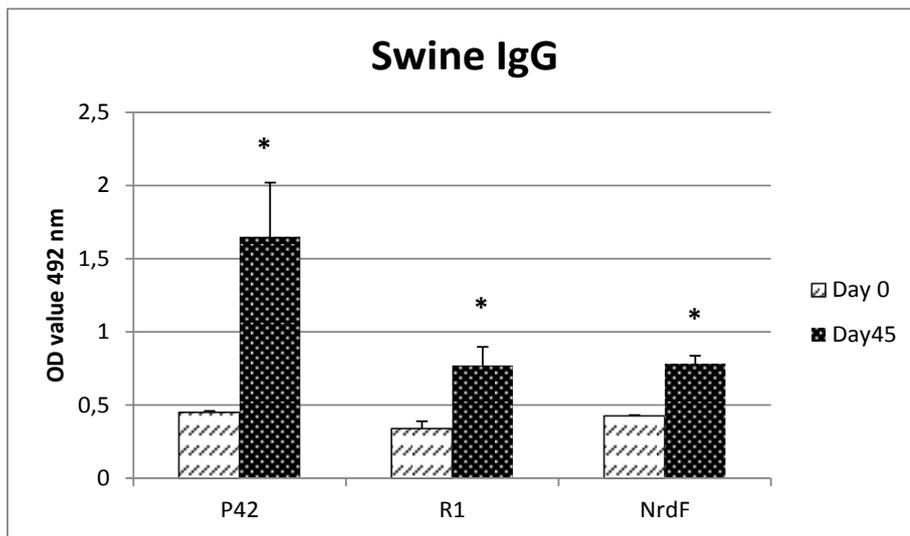


Figure 4. IgG absorbance at 492 nm of immunized pigs. Pigs were intramuscularly immunized with chimeric protein (rLTBR1P42NrdF) at days 0, 20 and 30. On days 0 and 45 serum was collected and the levels of anti-rP42, rR1 and rNrdF were determined by ELISA. ELISA antibody titers are presented as the mean from all the pigs on day 0 and 45. * $p < 0.05$ compared with the pigs serum on day 0.

Discussion

In the present study, a recombinant chimeric protein (rLTBR1P42NrdF) was produced that induced humoral and mucosal immune responses in mice by either IN or IM immunization and that a humoral immune response in pigs by IM immunization. Humoral and mucosal immune responses were characterized by the production of high levels of anti rR1, rP42 and rNrdF IgG in the sera and IgA antibodies in tracheobronchial lavage of vaccinated animals. The main strategy was to design a chimeric protein carrying different antigens from *M. hyopneumoniae* and an adjuvant molecule to increase the immunogenicity of the recombinant antigens. Some of these antigens (P97 and NrdF) have previously been shown to induce specific immune responses in pigs (Fagan et al; Shimoji et al., 2003), and the heat shock protein P42 has been considered a good immunogen in several studies (Simionatto et al., 2012; Chen et al., 2003).

Foreign antigens fused to recombinant LTB might constitute useful experimental mucosal vaccines (da Hora et al., 2011). LTB has been shown to be a potent mucosal adjuvant toward co-administered or fused unrelated antigens (Grassmann et al., 2012; Liu et al., 2011; Zhou et al., 2009). Foreign antigens fused to the LTB subunit can be delivered across the mucosal epithelium to the underlying mucosa-associated lymphoid tissue to stimulate the mucosal immune system by mediating the induction of antigen-specific serum IgG and mucosal IgA (Kim et al., 2011). Our results demonstrated that the presence of LTB, in either co-administered or fusion proteins, produced significantly higher levels of IgG and IgA antibodies in IN and IM immunization, although these levels differed between each antigen, with higher antibody levels against the rP42. However, no significant differences were found between co-administered or fusion proteins in some assays. In most assays, the fusion protein was more efficient. This corroborates with previous studies (Grassmann et al., 2012; Zhou et al., 2009), highlights the performance and advantages of chimeric proteins for use in vaccine development.

A major challenge for current vaccine development is the fact that many new subunit vaccines based on recombinant proteins are poorly immunogenic and therefore require to be formulated with adjuvants (Wilson-Welder et al., 2009). In this study the IgG levels in mice serum were significantly higher in the groups immunized IM when compared to the groups that received the same composition IN. Within the groups inoculated IM, the highest antibody response against rP42 and rR1 was found in the chimeric protein group. The antibody production against rNrdF was higher in the group that received antigens with adjuvant without fusion, however there was no difference between fusion and co-administration. Within the IN

immunized groups, significantly higher responses against the rP42 were observed in the chimeric group. The IgA antibody levels against each protein in the tracheobronchial lavage followed the same trend as the IgG levels in the serum of the groups inoculated by intranasal route, suggesting a correlation in the production of these two classes of antibodies. These results evidenced the effect of rLTB in either co-administered or fusion antigens as demonstrated by Grassmann and collaborators (2012), and confirmed the efficacy as a mucosal and systemic adjuvant as already described.

The mechanisms by which the enterotoxin-based adjuvant exerts immunomodulating effects are not fully characterized (da Hora et al., 2011). It has already been shown that LTB can increase both T helper type 1 and 2 (Th1 and Th2) associated cytokine response to LTB-fused antigens, once IFN- γ , IL-2, IL-4, and IL-10 were specifically and significantly induced by a multi-epitope vaccine against *Helicobacter pylori* (HUepi) fused to LTB, HUepi-LTB, in mice immunized orally (Zhou et al., 2009). Even though no cytokine production was measured in the present study it is commonly accepted that Th1 cells are preferentially involved in cell-mediated immunity, by the production of IgG2a antibodies, while Th2 cells are more effective in the regulation and support of B-cell responses, resulting in the production of IgG1 antibodies (Mosmann et al., 1989). The results of IgG1 and IgG2a seroconversion in mice immunized IM showed that the chimeric protein was able to induce both isotypes, significantly better than the other groups. In the groups inoculated IN, significant differences were only found in the response against rP42, with higher levels in the group inoculated with the chimeric protein. These results indicate a mixed Th1 and Th2 response, mainly against the rP42.

The chimeric protein was also able to induce specific IgG antibodies in pigs, demonstrating the same profile as in mice. Significant differences were detected between pre-immune and immune antibody levels in the serum against all antigens comprising the chimeric protein, with higher levels of antibodies against rP42. As for many infectious diseases, vaccination remains the approach for prevention and control of EP and the eradication of the pathogen has been proposed to depend on both the presence of serum antibody and an increase in cell-mediated immunity during mycoplasmal infection (Thacker et al., 2000), however, the actual contribution of both types of immune response in the protection against *M. hyopneumoniae* infections is not clearly defined (Okamba et al., 2010).

The production of anti P42-specific antibodies in the immunized mice and pigs confirmed that P42 induced a stronger immune response when compared with the other two antigens. This result corroborates with previous studies by our group, where the P42 was one

of the most immunogenic antigens among 34 different *M. hyopneumoniae* proteins evaluated (Simionatto et al., 2012). These results can be attributed to the fact that P42, as a heat shock protein, may contribute to an additional immunostimulatory effect. Heat shock proteins have been shown to present multiple B-cell and T-cell epitopes (Yagihashi et al., 1984) and have also been used as an adjuvant to enhance cell-mediated immune responses in DNA vaccines (Chen et al., 2000).

The Western blot characterization suggested that the recombinant chimeric protein retained antigenic epitopes present on native proteins. The chimeric protein was identified by specific sera against each subunit. In addition, serum of mice inoculated with the chimeric protein reacted with several *M. hyopneumoniae* strains. There was a weak reaction with the non-pathogenic J strain, indicating that the immune response induced by this protein is able to recognize the native antigens R1, P42 and NrdF. Immunoblotting results showed that the recombinant chimeric protein displayed good immunogenicity and antigenicity, and LTB retained the ability to bind to GM1.

In conclusion, the study described the design, expression and immunological response of a chimeric recombinant protein comprising three *M. hyopneumoniae* antigens and the adjuvant LTB. The chimeric protein exhibited antigenic profiles similar to those of the original proteins, and the antigens included induced an immune response in mice and in pigs. To our knowledge, this is the first demonstration of a multivalent subunit recombinant vaccine synthesized as a single molecule for *M. hyopneumoniae*. This approach may result in a formulation that could improve the protection against enzootic pneumonia in pigs. Further research will focus on the protective immunity of the vaccine against experimental *M. hyopneumoniae* in pigs.

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CHAPTER 3.3:

Production and characterization of recombinant transmembrane proteins from *Mycoplasma hyopneumoniae*

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Abstract

Mycoplasma hyopneumoniae is the etiological agent of swine enzootic pneumonia (EP), a chronic respiratory disease which causes significant economic losses to the swine industry worldwide. More efficient strategies for controlling this disease are necessary. In this study, we cloned 17 genes coding for transmembrane proteins from *M. hyopneumoniae*, among which six were successfully expressed in *E. coli* and had their immunogenic and antigenic properties evaluated. All proteins were immunogenic in mice and sera from naturally infected pigs reacted with the recombinant proteins, suggesting that they are expressed during infection. These antigens may contribute to the development of new recombinant vaccines and diagnostic tests against EP.

Keywords: *Mycoplasma hyopneumoniae*, swine enzootic pneumonia, transmembrane proteins.

Introduction

Porcine enzootic pneumonia, caused by *M. hyopneumoniae*, is a chronic respiratory disease affecting swine populations worldwide (Thacker, 2006). Vaccination is the most effective way to control EP, combined with hygiene and management procedures (Lin et al., 2003; Conceição et al., 2006; Maes et al., 2008). However, the vaccines currently used provide only partial protection (Thacker et al., 2000). These vaccines consist of inactivated whole-cell preparations (bacterins), and the vaccine is expensive (Chen et al., 2001), as *in vitro* growth of *M. hyopneumoniae* requires a rich medium and is a time consuming process (Thacker, 2006). Besides, more efficient and cheaper vaccines and improved diagnostic assays could contribute to the control of EP.

Some studies have tried to identify and characterize antigens from *M. hyopneumoniae* with vaccine and diagnostic potential (Castro et al., 2006; Chen et al., 2006; Conceição et al., 2006; Meens et al., 2009; Yang et al., 2005). However, only few recombinant antigens have been characterized so far. Bioinformatics tools can be used for predicting potential antigens *in silico*, narrowing down the universe to be tested. In addition, this approach has the advantage of revealing proteins independently of their abundance and without the need to grow the microorganism *in vitro* (Adu-Bobie, et al., 2003). The aim of this study was to identify, clone and characterize transmembrane proteins, targets for many pharmaceuticals in use today, from *M. hyopneumoniae* which may be useful in immunodiagnosis and/or vaccination against EP.

Materials and methods

Strains and growth conditions

M. hyopneumoniae strain 7448 was gently provided by Centro Nacional de Pesquisa em Suínos e Aves (CNPISA – Embrapa, Concórdia, SC). Genomic DNA was extracted with GFX genomic blood DNA purification kit (GE Healthcare). *Escherichia coli* TOP 10 (Invitrogen) and *E. coli* BL21 (DE3) RIL (Stratagene) were grown at 37 °C in Luria Bertani (LB) medium supplemented with kanamycin (100 µg.ml⁻¹), as required.

In silico selection of open reading frame and primer design

One hundred and thirty six open reading frame (ORF) belonging to the group of transmembrane proteins of *M. hyopneumoniae* strain 7448 (GenBank accession number [NC007332](http://www.ncbi.nlm.nih.gov/nuccore/NC007332)), previously characterized (<http://www.genesul.incc.br>), were analyzed by bioinformatics software. To determine the signal peptide, the SignalP software was used. Vector NTI 10 (Invitrogen) was used to identify sequences with predominantly hydrophilic regions, and with no more than three TGA tryptophan codons. Primers were designed to

amplify the selected sequences. The forward primer included the sequence CACC for directional cloning into expression vector pET200/D-TOPO® (Invitrogen). The primers used in this study are shown in Table 1.

Amplification and cloning of coding sequences

The selected sequences were amplified by PCR using Mastercycle Gradient (Eppendorf) thermocycler, with the following program: 95 °C for 7 min followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 68 °C for 1 min, with a final extension of 68 °C for 7 min. The reactions were performed in a final volume of 25 µl, containing 50 ng of template DNA, 10 pmol of each primer, 1 × enhancer, 1 × enzyme buffer, 2.5 mM MgCl₂, 0.2 mM of dNTPs and 2.5 units of Platinum Pfx DNA Polymerase (Invitrogen). Sequences that contained TGA codons were submitted to site-directed mutagenesis according to Simionatto et al. (2009). PCR products were cloned in Champion pET200/D-TOPO® (Invitrogen) expression vector following the manufacturer's instructions. *E. coli* Top 10 competent cells were transformed by electroporation with the ligation product, as described by Sambrook & Russell (2001). The recombinant clones were selected and plasmid DNA was extracted and characterized by digestion with restriction enzymes and DNA sequencing using a MegaBACE 500 DNA sequencer and the Dynamic ET-terminator technology (GE Healthcare).

Expression, solubility testing and purification of recombinant proteins

Recombinant plasmids were transformed into *E. coli* BL21 (DE3) Ril. The resulting colonies were used to inoculate 10 ml of LB broth supplemented with 100 µg.ml⁻¹ of kanamycin and grown in shaker (37 °C, 250 rpm). When the log phase was reached, expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Two fractions of 500 µl, one collected before and another after induction, were centrifuged at 14,000 × g for 1 min. The sediment was used to verify expression of recombinant proteins by 12% SDS-PAGE. The recombinant protein solubility test was performed using 2 ml of the sample induced and subjected to centrifugation of 14,000 × g for 5 min. A volume of 500 µl of 1 × phosphate buffered saline (PBS) was used to suspend the pellet and the cells were lysed by six cycles of sonication (15 s, 20 kHz). The soluble and insoluble fractions were separated by centrifugation at 10,000 × g for 5 min at 4 °C. Both fractions were processed and subjected to electrophoresis in 12% SDS-PAGE.

A clone of each recombinant plasmid was used to inoculate 15 ml of LB containing 100 µg.ml⁻¹ of kanamycin and grown overnight in shaker (37 °C, 250 rpm). This culture was used to inoculate 500 ml of LB broth supplemented with kanamycin under the same conditions described above. After 3 h of induction, the culture was subjected to centrifugation

at $10,000 \times g$ for 20 min at 4 °C, the supernatant discarded and the pellet subjected to two successive washes in 60 ml of PBS and centrifuged under the same conditions. Then, it was suspended in 60 ml of lysis buffer (8 M urea, 20 mM Na_2HPO_4 , 0.5 M NaCl, 5 mM Imidazole). The cells were lysed by six cycles of sonication (15 s, 20 kHz) and submitted to orbital shaking of 60 rpm for 16 h at 4 °C. After this, the lysate was centrifuged again at $10,000 \times g$ for 1 h at 4 °C, the supernatant containing the protein was collected, filtered through a membrane filter of 0.8 μm (Millipore) and used for purification. The purification of recombinant proteins was done by affinity chromatography using HisTrap™ HP 1 ml columns prepacked with precharged Ni Sepharose™ using the ÄKTAprime™ automated liquid chromatography system (GE Healthcare). Fractions containing recombinant proteins were identified by SDS-PAGE and quantified by BCA™ Protein Assay (Pierce) following the manufacturer's instructions.

Inoculation of mice with recombinant proteins

Animal experiments were carried out according to the guidelines of the Ethics Committee in Animal Experimentation of the Federal University of Pelotas. Female and male BALB/c mice aged 7 to 8 weeks were used in the experiments. Eight groups of mice with five animals each, were inoculated intramuscularly (IM) with 50 μg of recombinant proteins, one per group in 15% aluminum hydroxide adjuvant on day zero. A group inoculated with a commercial bacterin was used as positive control and another group received 100 μL of PBS with the same adjuvant, as negative control. Each animal was boosted with the same dose 21 days after the first inoculation. Blood samples were collected from the retro-orbital sinus at 0, 21, 42, 63, 84, and 105 days post inoculation (DPI). The sera were processed and stored at -20 °C until tested. At day 105, mice were euthanized.

Evaluation of immune response

ELISA using recombinant proteins as antigen

Specific antibodies against recombinant proteins in sera from immunized mice were assayed by indirect ELISA. Microtiter plates were coated with 100 μL of each recombinant protein at a concentration ranging from 5 to 50 ng/well in 0.1 M sodium carbonate buffer (pH 9.6), at 4 °C overnight. The plates were washed three times with PBS (pH 7.4) with 0.05% (v/v) Tween 20 (PBST) and incubated with 200 μL of 5% blocking buffer at 37 °C for 2 h. After washing with PBST, wells were incubated with mice sera, in triplicate, of each individual animal, diluted 1:50 in blocking buffer, at 37 °C for 1 h. After washing with PBST, goat anti-mouse peroxidase conjugate (Sigma Aldrich) diluted 1:6000 was added and the plate was incubated at 37 °C for 1 h. The reactions were developed with *o*-

phenylenediaminedihydrochloride (Sigma) and hydrogen peroxide, after PBST washes. The color reaction was allowed to develop for 15 min before being stopped with 50 μ l of sulfuric acid 1 N. Absorbance was determined at 492 nm with a microplate reader (Multiskan MCC/340, Titertek Instruments). Mean values were calculated from sera samples assayed in triplicate.

ELISA using extract of M. hyopneumoniae 7448 strain as antigen

An ELISA was performed with sera from mice immunized with recombinant proteins to verify if the antibodies induced by these antigens were able to recognize *M. hyopneumoniae* derived proteins. For this, 1 μ g of crude extract of *M. hyopneumoniae* strain 7448, dissolved in 0.1 M sodium carbonate buffer (pH 9.6), was added to each well of microtiter plates. The plates were incubated overnight at 4 °C, then incubated at -70 °C for 2 h and thawed at room temperature for 30 min. Then, the same procedure described above was used, except that sera from animals of each group were pooled before testing. Mean values were calculated from pooled sera samples assayed in triplicate.

Western blot with M. hyopneumoniae derived proteins

In order to evaluate the ability of antibodies from mice immunized with recombinant proteins to recognize derived proteins from *Mycoplasma* sp., lysates of *M. hyorhinis*, *M. hyopneumoniae* 7448, 7422 and J^T strains, were used in Western blot analysis. Samples were solubilized in sample buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, 2% SDS) and separated by 10% SDS-PAGE. Mycoplasma derived proteins were transferred onto nitrocellulose membranes (GE Healthcare) using a mini transblotter, following the manufacturer's instructions (Bio-Rad). The membrane was blocked overnight at 4 °C in 5% nonfat dried milk-PBST and probed with pooled sera from each group of mice (diluted 1:50 in blocking buffer). Specific antibodies were detected with goat anti-mouse peroxidase conjugate. Immunoreactive protein bands were detected using 3,3'-diaminobenzidinetetrahydrochloride (DAB) and 0.015% (v/v) hydrogen peroxide in PBS. Sera from mice immunized with PBS and bacterin were used as negative and positive controls, respectively.

Antigenicity of the recombinant antigens

Serum samples from SPF pigs and from naturally infected pigs (from a commercial herd with clinical signs and pathological lesions compatible with EP) were used to evaluate the reactivity of recombinant antigens with antibodies produced during infection. The sera were tested by Western blot and ELISA.

Western blot

Purified recombinant proteins were solubilized in sample buffer and submitted to 12% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (GE Healthcare) using a mini transblotter (Bio-Rad) following the manufacturer's instructions. Membranes were incubated in 5% blocking buffer overnight at 4 °C. After washing with PBST, membranes were incubated individually with adsorbed sera (diluted 1:100 in blocking buffer) from SPF (1), pool of 3 immunized pigs, or 10 naturally infected pigs, for 2 h at 37 °C. To reduce the background, the sera were previously adsorbed in *E. coli* extract. Membranes were washed with PBST (3 × 5 min per wash) and incubated for 1 h at 37 °C with rabbit anti-pig IgG peroxidase conjugate, diluted 1:6000 in blocking buffer. Membranes were washed in PBST (3 × 5 min per wash). Immunoreactive protein bands were detected using 3,3'-diaminobenzidinetetrahydrochloride (DAB) and 0.015% (v/v) hydrogen peroxide in PBS.

ELISA

To assess the antigenicity of recombinant proteins by ELISA, porcine sera from animals naturally infected (n=22) and SPF (n=5) were used. Sera from naturally infected pigs had antibody titers checked by cell ELISA. Microtiter plate were coated with 5 to 50 ng of each recombinant protein in 0.1 M sodium carbonate buffer (pH 9.6), at 4 °C overnight. The plates were washed three times with PBST, and incubated with 200 µL of 5% blocking buffer, at 37 °C for 2 h. After washing with PBST, wells were incubated with swine sera, in triplicate, diluted 1:100 in blocking buffer, at 37 °C for 2 h. After washing with PBST, wells were incubated with rabbit anti-pig IgG peroxidase conjugate, diluted 1:6000, at 37 °C for 1 h. The reactions were developed with *o*-phenylenediaminedihydrochloride (Sigma) and hydrogen peroxide, after PBST washes. The color reaction was allowed to develop for 15 min and stopped with the addition of 50 µL of 1 N H₂SO₄. Absorbance was determined at 492 nm with a microplate reader. Mean values were calculated from sera samples assayed in triplicate.

MHC-I antigenic peptide processing prediction of characterized recombinant antigens

Peptides were predicted using MAPPP (Hakenberg et al., 2003) with the SYFPEITHY matrix (available at <http://www.mpiib-berlin.mpg.de/MAPPP/expertquery.html>) which combines existing prediction tools for proteasomal processing and MHC class I anchoring. We considered the epitopes when the MHC, from human (HLA) and mouse (H2), bound epitope evolved from a proteasomal cleaved fragment with exactly the same size, considering all nonamers with potential binding for any of the alleles available. For each nonamer and allele, the program produced a binding score that corresponded to the estimate of half-time of

disassociation for a given peptide. That score was expressed as a percentage of the maximum score possible for a given allele.

Statistical analysis

The Student's *t*-test was used to determine significant differences ($p < 0.05$) in ELISA with recombinant proteins using pig sera and cell ELISA of mice immunized with recombinant proteins. GraphPad Prism 4 software systems (GraphPad Software) were used to perform the statistical analyses.

Results

Selection of ORFs

The following criteria were used for selecting among ORFs encoding predicted transmembrane proteins to be cloned and expressed in *E. coli*: proteins previously characterized as virulence factors or orthologous to virulence factors characterized in other pathogens; hydrophobicity properties of the protein and with no more than three tryptophan (TGA) codons. Preference was given to hydrophilic regions without TGA codons. A total of 17 ORFs belonging to the group of transmembrane proteins of *M. hyopneumoniae* met these criteria and were selected. One of these had two selected regions, C and N-terminal. A total of 18 fragments were selected and primers were designed (Table 1).

Table 1. Identification of selected ORFs and primer sequences.

N°	Annotation	Selected Fragment (aa)	Primer sequences
1	MHP0019	238 - 665	<i>For</i> * <u>CACCGGATCCGGCAAATTCAA</u> AACTGGAC <i>Rev</i> GGGAAGCTTTCACAAAACCCCAATCATTTTC
2	MHP0020	16 - 298	<i>For</i> <u>CACCGGATCCATTTTTAAAAA</u> ATTAATCTTGTA <i>Rev</i> GGGAAGCTTTCATATTGAAATTGAATCTAATT
3	MHP0241	20 - 195	<i>For</i> <u>CACCGGATCCTTTCTTGCTTTTT</u> TGACTT <i>Rev</i> CCCAAGCTTTCACTCAAGTTGAGCTTTTTTATC
4	MHP0248	248 - 748	<i>For</i> <u>CACCGGATCCCTAGGACAGTTT</u> GCAATAATTATTGT <i>Rev</i> CCCAAGCTTTCATTTATCTTTTTTATCATCAATAATTC <i>For mut**</i> GGATTTTgGAATGgTTTTGgGGCAAATTA <i>Revmut</i> TTTGCCcCAAAAcCATTcCAAAATCCTTG
5	MHP0265	123 - 311	<i>For</i> <u>CACCGGATCCTATTTAGTAAA</u> ACCGACTGTTT <i>Rev</i> CCCAAGCTTTCACTTTGGCAAATGCGAAA
6	MHP0328	1 - 172	<i>For</i> <u>CACCGGATCCATGACCAAATTT</u> GCAAAA <i>Rev</i> CCCAAGCTTTCAGCTAGTAACATGTTCTTTCCA
7	MHP0332	56 - 264	<i>For</i> <u>CACCGGATCCTATGCTTCAAAT</u> TTTAGT <i>Rev</i> CCCAAGCTTTCACTTATTAGTAGATC
8	MHP0344	1 - 166	<i>For</i> <u>CACCGGATCCATGAAAATAAAA</u> AAACAAA <i>Rev</i> CCCAAGCTTTC AAGGGAATGGAGTACC
9	MHP0383	1 - 257	<i>For</i> <u>CACCGGATCCATGATTCAAAT</u> TCGTAATGTT <i>Rev</i> CCCAAGCTTTCATATTTTTTTGCTAATATCAGC
10	MHP0414 N	904 -1199	<i>For</i> <u>CACCGGATCCTTCAATAGAAA</u> AGAATATTATACTATTC <i>Rev</i> GGGAAGCTTTCATTTATTA ACTAAATTATGAAA
11	MHP0414 C	67-284	<i>For</i> <u>CACCGGATCCATAAACAACCC</u> AAATATTTATTC <i>Rev</i> GGGAAGCTTTTAACTATTATACCTTTTAATTTTCC
12	MHP0443	31 - 512	<i>For</i> <u>CACCGGGTACCGATTGATTTTT</u> GTCTTTTTTG <i>Rev</i> CCCAAGCTTTCAGATTCTACCATTAATCTTT
13	MHP0480	258 - 524	<i>For</i> <u>CACCGGATCCAATGACTGGAT</u> CAAAAACCTA <i>Rev</i> CCCAAGCTTTCATGCTTTATTATTATCGATC
14	MHP0487	2 - 284	<i>For</i> <u>CACCGGATCCGATAAAAAA</u> AGTTCTTTTCTTTT <i>Rev</i> CCCAAGCTTTCAAAAAACTCTTGATTTTACGAT
15	MHP0489	576 - 744	<i>For</i> <u>CACCGGATCCCAGCAAAC</u> TTATAATTCTC <i>Rev</i> CCCAAGCTTTCAGGAATAATCAAGAACGA
16	MHP0544	31- 386	<i>For</i> <u>CACCGGATCCATTAATCAGTT</u> GATTAATTCCTTTT <i>Rev</i> GGGAAGCTTTCAGCATGAATTTATTTTTTTTATTA
17	MHP0596	110 - 379	<i>For</i> <u>CACCGGATCCCTTAGCCAAA</u> ATCCACTAC <i>Rev</i> CCCAAGCTTTC AATTTTCGTTTTTATC
18	MHP0643	131 - 345	<i>For</i> <u>CACCGGATCCTTTTTTATCGG</u> TTTTTTTTAT <i>Rev</i> GGGAAGCTTTCAGTTAATCGTTACAATATTTG <i>For mut</i> CCAGTCTGgAAAGAGAATAATT <i>Revmut</i> ATTCTCTTTcCAGACTGGAAT

*CACC: Sequence of nucleotides added for directional cloning in the vector Champion pET 200/D-TOPO[®] (Invitrogen).

** Pair of primers used for mutation from TGA to TGG codon.

Amplification, cloning and characterization of genes

All 18 selected fragments were amplified by PCR. Two fragments that had TGA tryptophan codons were submitted to site-directed mutagenesis and had the TGA replaced by

TGG codons. The PCR amplified sequences were cloned into Champion pET200/D-TOPO® (Invitrogen) and transformed into *E.coli* TOP10 electro competent cells. Out of the 18 amplicons, 16 were successfully cloned and characterized (Table 2).

Expression and purification

Clones characterized by restriction enzyme showing the insert of the expected size were subjected to transformation by heat shock in the expression strain *E. coli* BL21 (DE3) RIL. Before being expressed in large scale, the recombinant proteins were assessed in small volume growth for their expression and solubility. All the expressed proteins were insoluble and had to be solubilized with a denaturing agent containing urea. As the expression vector used (pET200/D-TOPO) allows the fusion of 6 histidine residues in the N-terminal of recombinant proteins, they were purified by affinity chromatography. Among the 16 clones, 11 recombinant proteins were expressed and six were successfully purified in concentrations suitable for immunizations and other tests (Fig. 1). The remaining five recombinant proteins were expressed in low levels and purification was not successful. The yield of recombinant proteins ranged from 4.4 to 6.6 mg l⁻¹ of culture growth.

Table 2. Identification of genes that code for proteins selected, features, size and results.

Annotation	Features	Molecular Mass (kDa)	Cloning	Expression	Purification	Mice immunization	Antigenicity with pig sera (Western blot)
MHP0019	ABC transporter	50	Yes	No	No	No	-
MHP0020	ABC transporter	35	Yes	Yes	No	No	-
MHP0241	Translocase	22	Yes	Yes	No	No	-
MHP0248	Hypothetical protein	60	Yes	No	No	No	-
MHP0265	ABC transporter	23	Yes	No	No	No	-
MHP0328	Hypothetical protein	21	Yes	Yes	Yes	Yes	+ ^b
MHP0332	Serine protease	25	Yes	Yes	Yes	Yes	+
MHP0344	Hypothetical protein	19	Yes	No	No	No	-
MHP0383	ABC transporter	29	Yes	No	No	No	-
MHP0414 C	Putative ICEF-II ^a	31	Yes	Yes	Yes	Yes	-
MHP0414 N	Putative ICEF-II	26	Yes	Yes	Yes	No	+++ ^c
MHP0443	Hypothetical protein	56	No	No	No	No	-
MHP0480	Hypothetical protein	31	Yes	Yes	Yes	Yes	-
MHP0487	Putative Mg transport	32	No	No	No	No	-
MHP0489	Hypothetical protein	20	Yes	Yes	Yes	Yes	-
MHP0544	Hypothetical protein	41	Yes	Yes	No	No	-
MHP0596	Hypothetical protein	31	Yes	Yes	Yes	Yes	+++
MHP0643	Hemolysin C	26	Yes	Yes	No	No	-

a: ICEF-II: integrative conjugal element – II; b: React with one of ten sera evaluated; c: React with seven of ten sera evaluated.

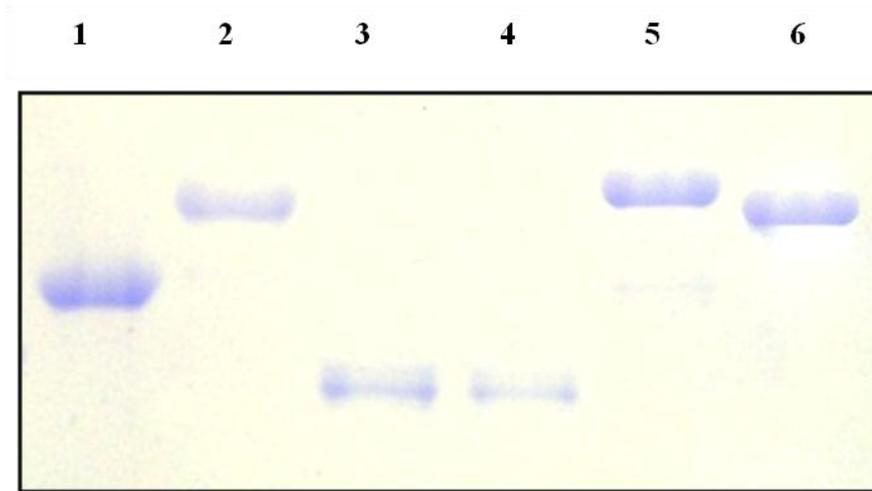


Figure 1. Twelve percent SDS-PAGE of six recombinant proteins purified by Ni-Sepharose affinity chromatography. Lane 1, MHP0332 (25 kDa); Lane 2, MHP0480 (31 kDa); Lane 3, MHP0328 (21 kDa); Lane 4, MHP0489 (20 kDa); Lane 5, MHP0596 (31 kDa); Lane 6, MHP0414 C (31 kDa).

Systemic humoral immune response

The ability of recombinant proteins, produced from ORF, to stimulate specific antibodies after immunization of mice was determined by an indirect ELISA. Groups of animals were identified as A: negative control (saline + adjuvant), B: MHP0332, C: MHP0414 C, D: MHP0480, E: MHP0489, F: MHP0596, G: MHP0328, and group H: animals inoculated with bacterin. All recombinant proteins were able to induce a specific immune response statistically significant when compared with the control group. The highest immune response was induced by the protein produced from ORF MHP0328, while the MHP0596 induced the lowest immune response among all proteins tested. Figure 2 shows the responses of all proteins up to the day 84 after immunization.

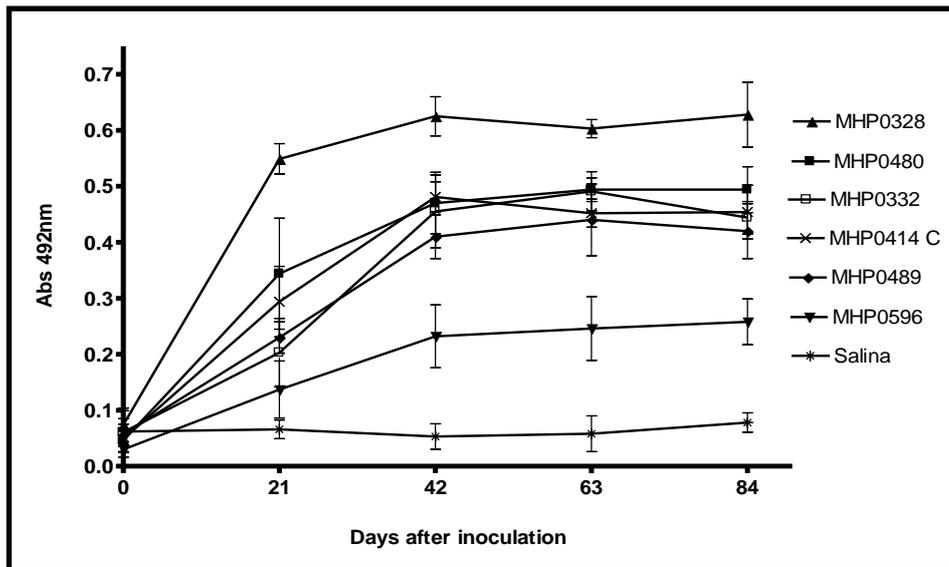


Figure 2. Antibody responses of mice immunized with recombinant proteins determined by indirect ELISA. Reactivity of the serum diluted 1:50, in triplicate. Goat anti-mouse peroxidase conjugate (diluted 1:4000) was used as the secondary antibody. The data represent the mean OD₄₉₂. Vertical lines represent standard deviation.

Sera from inoculated mice were also evaluated by ELISA against the whole cell extract of *M. hyopneumoniae* strain 7448. Proteins derived from ORFs MHP0480 and MHP0328 showed statistically significant differences in absorbance values when sera collected on day 84 after immunization was compared to the pre-immune serum (Fig. 3).

Western blot analysis was carried out with mouse sera raised against six recombinant proteins and revealed that antibodies were able to recognize mycoplasma derived proteins. Five recombinant proteins induced antibodies that reacted with derived proteins from *M. hyopneumoniae* 7448, 7422, J^T strains and *M. hyorhinis*. The MHP0480 recombinant protein induced antibodies that appear not to cross-react with *M. hyorhinis* (data not shown), a common secondary invader in swine pneumonia. Sera from mice inoculated with saline did not recognize mycoplasma proteins, while sera from mice inoculated with bacterin recognized several mycoplasma proteins.

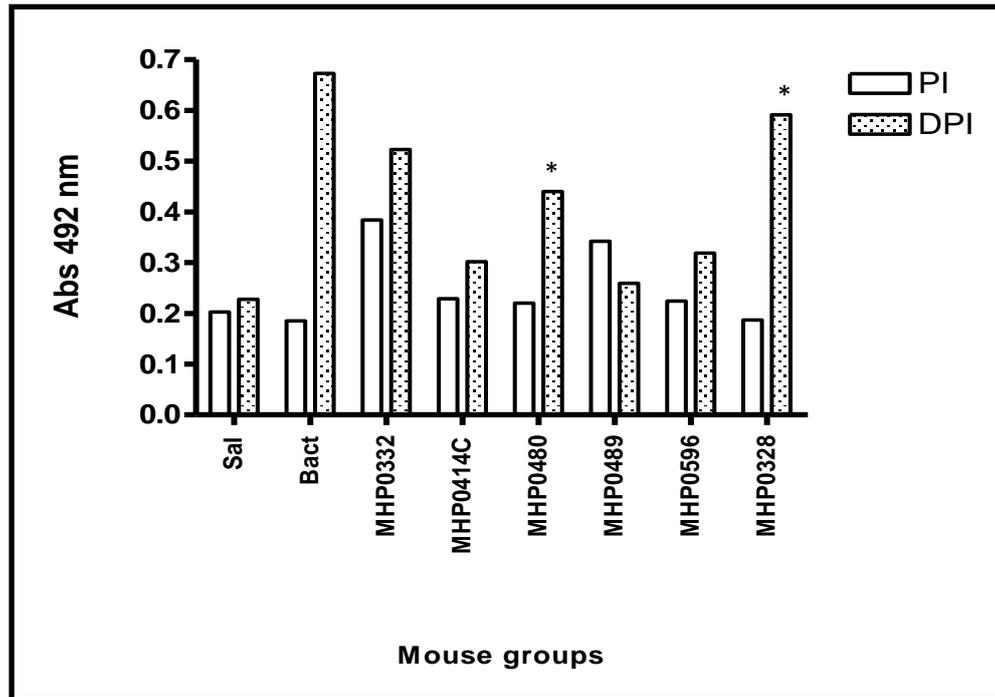


Figure 3. ELISA reactions using extract of *M. hyopneumoniae* as antigen. Detection of the antibodies in sera from mice immunized with recombinant proteins (diluted 1:20). Goat anti-mouse peroxidase conjugate (diluted 1:4000) was used as the secondary antibody. PI, pre-inoculation mouse sera; DPI, sera of mice obtained 105 days post-inoculation; Sal and Bact, are the negative group inoculated with PBS and positive group immunized with bacterin, respectively. The data represent the mean OD₄₉₂ of sera (105 DPI) from immunized mice. Asterisks indicate significant statistical differences ($p < 0.05$ as compared to PI sera) using Student's *t*-test.

Antigenicity of the recombinant antigens

Western blot analysis revealed that sera from some naturally infected animals were able to recognize four recombinant proteins (Table 2). Proteins produced from ORFs MHP0414 N (not used in the immunization tests) and MHP0596 were the most antigenic, as they were recognized by seven out of 10 sera tested (data not shown).

ELISA demonstrated that sera from naturally infected pigs were able to recognize the recombinant proteins (Fig. 4), indicating that these proteins are expressed during infection and they induce a humoral immune response.

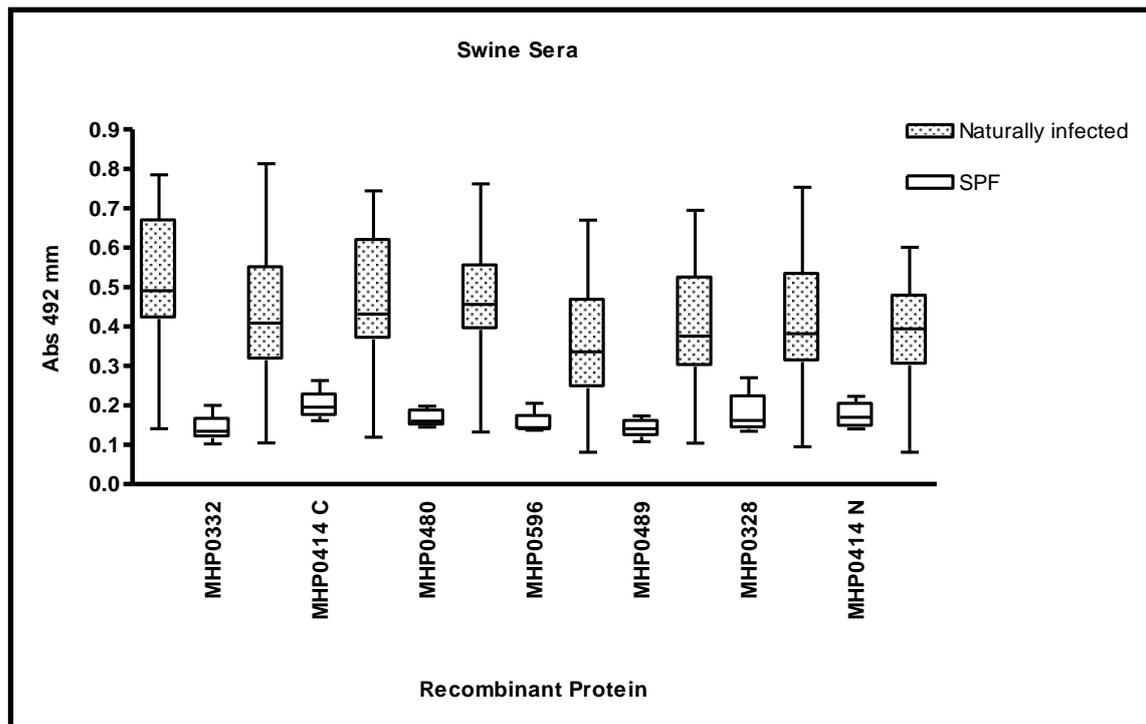


Figure 4. ELISA of recombinant proteins against naturally infected and SPF pig sera (diluted 1:100). Anti-pig IgG-peroxidase conjugate (diluted 1:6000) was used as the secondary antibody. All recombinant proteins showed significant statistical difference ($P < 0.05$ as compared to SPF sera). The data represent the analyses of 5 SPF and 22 naturally infected pig sera. Absorbance values were statistically analyzed with a paired t-test using GraphPad Prism 4.0 software ($P < 0.05$). Boxes represent the interquartile range in the middle 50% of absorbance values. Whiskers represent the minimum and maximum values.

MHC-I antigenic peptide processing prediction of characterized recombinant antigens

We considered the epitopes when the MHC bound epitope evolved from a proteasomal cleaved fragment with exactly the same size. All recombinant proteins evaluated contain at least one epitope that have the greatest probability to be processed and finally presented on the cell surface. It is expected that those peptides which have higher affinity for HLA and H2 molecules are more likely to be recognized by receptors of specific T cells. The peptides representing predicted dominant MHC class I epitopes from recombinant antigens are shown in Table 3.

Table 3. MHC bound epitope evolved from a proteasomal cleaved fragment with exactly the same size from characterized recombinant antigens of *M. hyopneumoniae*.

Annotation	Epitope	Epitope Position	MHC type	Cleavage Probability	MHC Binding Score
MHP0328	TMEDFANKL	156	HLA_A_0201	0.9997	0.6111
	IREPFAKQL	10	HLA_B_2705	1.0000	0.6486
MHP0332	QFLDNYYYYYL	127	H2_Db	1.0000	0.7273
	QFLDNYYYYYL	127	H2_Kd	1.0000	0.7143
MHP0414 C	KRNQNIFDQL	47	H2_Db	1.0000	0.7273
	QLPKKIINL	55	HLA_A_0201	0.9719	0.6944
	SNLPNIKDL	149	H2_Db	0.9966	0.6857
MHP0480	LLDFSLNQL	13	HLA_A_0201	0.9718	0.7222
	NLSSEIINL	73	HLA_A_0201	1.0000	0.7059
MHP0489	SYYPYFDSRA	54	H2_Kd	1.0000	0.7143
	RYQFGQGHKL	66	H2_Kd	0.9847	0.8214
	CLKYRAFLL	93	HLA_B8	1.0000	0.8140
	KRNLSIDYL	115	HLA_B_2705	0.9990	0.7297
	YLLKIINYEYV	122	HLA_A_0201	1.0000	0.7941
MHP0596	LSQNPLPEL	0	H2_Ld	1.0000	0.7097
	ILSIITKTKL	95	HLA_A_0201	1.0000	0.7059
	SYNKHKMQQL	208	H2_Kd	1.0000	0.8571

Discussion

Production of recombinant proteins of *M. hyopneumoniae* in *E.coli* has been suggested as an alternative not only for vaccine production, but also for diagnostic tests of EP (Simionatto et al., 2010). Vaccination has been an important preventive tool in controlling infection by *M. hyopneumoniae* (Reynolds et al., 2009). However, commercial vaccines confer only partial protection (Haesebrouck et al., 2004). The sequencing of four strains of *M. hyopneumoniae* (Liu et al., 2011; Minion et al., 2004, Vasconcelos et al., 2005) has allowed the use of a genomic approach in the production of recombinant subunit vaccines, named reverse vaccinology (Rappuoli, 2000). This approach may contribute for the development of new and more effective vaccines against EP and in identification of proteins that are expressed during infection and can thus be used in diagnostic tests.

In this study, recombinant antigens belonging to the group of transmembrane proteins of *M. hyopneumoniae* were evaluated as candidates for vaccine and for use in sero diagnosis of EP. Transmembrane proteins play critical roles in cellular metabolism, participating in processes such as ion transport, nutrient uptake, signal transduction, and intercellular communication. As evidence of the essential functions of these proteins, more than half of all drug targets have been shown to be transmembrane proteins, and the analysis of the interactions of transmembrane proteins and their ligands is one of the most promising avenues for the discovery of new drug candidates as well vaccine targets (Yoshino et al., 2010; Janulczyk and Rasmussen, 2001).

The proteins evaluated in this study were first assessed through bioinformatics software to confirm their location, presence of potential immunogenic epitopes and to evaluate the number of TGA codons, which in *M. hyopneumoniae* code for tryptophan, but in other organisms are stop codons. The directional cloning strategy used was effective, allowing the cloning of all selected fragments, despite the fact that the genome of *M. hyopneumoniae* is rich in AT (72%) (Vasconcelos et al., 2005). The recombinant proteins were expressed in insoluble form, although hydrophilic regions of the proteins were predominantly selected. This fact can be justified because transmembrane proteins are generally expressed at low levels and have hydrophobic domains. There are considerable challenges to be overcome for the stable expression of transmembrane proteins, as many of them have cytotoxic effects when overexpressed (Wagner et al., 2007), leading to formation of inclusion bodies (Georgiou and Segatori, 2005).

Six recombinant proteins were purified in sufficient amounts for further analyses. Immunization of mice with the recombinant proteins revealed that all of them induced a

specific humoral immune response. The hypothetical protein, produced from ORF MHP0328 induced the highest humoral immune response, assessed by both, ELISA and cell ELISA, suggesting that this antigen may be a candidate for vaccine formulations. None of the recombinant proteins evaluated in this study showed response when reacted with the serum of the group of animals inoculated with the commercial vaccine (data not shown). This indicates that these antigens are not expressed by the vaccine strain. The absence of immunogenic antigens in the vaccine strains could be the explanation for the limited efficiency of commercial bacterins.

The mechanisms responsible for immune protection against *M. hyopneumoniae* are complex and not well understood yet (Reynolds et al., 2009). Thacker et al. (2000) suggested that eradication of the pathogen is dependent on the presence of antibodies in the serum and on cell-mediated immunity during infection. Therefore, promising antigens for formulation of vaccines should induce both types of response. In this study, all antigens were able to induce a humoral immune response when evaluated in mice. Although the cellular immune response induced by these recombinant antigens was not evaluated, they have one or more epitopes binding to MHC-I. *In silico* T-cell epitope mapping using computational models has been used in cancer immunity (Depilet et al., 2007), autoimmunity (Honeyman et al., 1998), infectious diseases (Brusic et al., 2001), allergy (De Lalla et al., 1999) and is currently emerging as a novel approach for the study of genome-based peptide vaccines (De Groot, 2006).

ELISA demonstrated that all proteins showed a significant reaction with sera from naturally infected pigs. The recognition of the recombinant proteins by serum from pigs naturally infected indicates that these proteins are expressed during infection, suggesting that they have potential to be used in serological diagnostic tests. However, when these proteins were tested by Western blot, four were recognized by antibodies in the sera, two of them, MHP0596 (hypothetical protein) and MHP0414 N (putative ICEF-II), were recognized by seven out of ten sera evaluated. From all recombinant proteins evaluated in this study, only the MHP0480 did not react with *M. hyorhinis*, a common secondary invader found in the respiratory tract of pigs, suggesting that it could be used for immunodiagnosis of EP.

Production of experimental recombinant vaccines against EP has been pursued by many scientists. Some of these vaccine candidates have shown promising results (Chen et al., 2003; Fagan et al., 1996; Okamba et al., 2010; Shimoji et al., 2003). However, just two antigens have been evaluated, and they were able to confer only partial protection for pigs. This can be attributed to the fact that they were formulated with a single antigen. A multiple

antigen vaccine has the potential to offer more adequate protection (Chen et al., 2008; Okamba et al., 2010).

In the present study it was demonstrated that six transmembrane proteins of *M. hyopneumoniae*, not previously characterized, are immunogenic and expressed during infection, suggesting that these antigens have potential to be used as vaccine against EP, as well as in diagnostic tests. Although the immune response of mice cannot be extrapolated to other species and no direct correlation has been found between antibody concentration and protection against *M. hyopneumoniae* challenge (Djordjevic et al., 1997), the results warrant further investigation in challenge experiments in pigs.

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CHAPTER 4.

GENERAL DISCUSSION

General Discussion

M. hyopneumoniae is the causal agent of enzootic pneumonia, a contagious pulmonary disease of swine characterised by a dry, non-productive cough, reduced growth rate and poor feed conversion efficiency, that represents a costly problem for the pig industry worldwide (Thacker and Minion, 2010). Factors such as management practices, housing conditions, secondary infections and the use of antimicrobials and commercial bacterin-based vaccines can influence the severity of mycoplasmal respiratory disease in pig herds (Maes et al., 2008).

In addition, the virulence status of *M. hyopneumoniae* isolates may also affect the clinical pattern of infection, and thus the protective efficacy of vaccination may vary depending on the virulence of the strain causing infection (Vicca et al., 2003; Villarreal et al., 2011). Notwithstanding the efforts and control strategies adopted to rear high health pig herds, *M. hyopneumoniae* continues to be a widely spread organism in pig populations, which renders eradication of this respiratory disease a very difficult and frustrating task.

In the present thesis, the immune responses induced by a commercially available adjuvanted bacterin against *M. hyopneumoniae* were investigated, and a new vaccine based on recombinant proteins of *M. hyopneumoniae* was developed.

Immune response induced by a commercial bacterin in pigs

The currently used vaccines consist of adjuvanted, inactivated, whole-cell preparations and have been proven extensively to be effective in reducing the clinical symptoms and lung lesions, and in decreasing performance losses associated with *M. hyopneumoniae* infections (Maes et al., 2008). However, they provide only partial protection (Villarreal et al., 2011; Vranckx et al., 2012), and the mechanism of protection is not yet fully understood. The study reported in the chapter 3.1 investigated the mechanisms by which a commercial *M. hyopneumoniae* bacterin, applied once intramuscularly in piglets of 7 days old, can induce an immune response against EP.

Few information is available with regard to immune mechanisms induced by intramuscular vaccination with inactivated *M. hyopneumoniae* vaccines. The mode of action of the vaccine was evaluated by a number of immunological assays designed to measure local and systemic humoral and cellular immune responses. Understanding the immune response induced by the current vaccines can give better insights in the mechanisms involved in the

protection against EP and might be useful to develop new and more efficient strategies of vaccination. Immunological assays were performed on blood, bronchoalveolar lavage (BAL) fluid and different lymphoid tissues (lung, bronchial lymph nodes and spleen) of vaccinated and non-vaccinated pigs.

In agreement with previous reports, we observed individual variations in the number of T cell subsets (Borghetti et al., 2006; Kick et al., 2010). In both vaccinated and non-vaccinated pigs, the percentage of CD8⁺ T cells increased with age of the pigs, showing that different T cell subsets may be present in the peripheral blood and secondary lymphoid tissues. In most tissues, the percentage of CD8⁺ T cells was highest in the vaccinated group at 58 days of age. It might be that vaccination modulates the immune response induced by a *M. hyopneumoniae* infection. CD8⁺ T cells may modulate the inflammatory response through the production of IFN- γ and other cytokines, minimizing damage due to the CD4⁺ T (Th) cell mediated inflammatory responses (Jones and Simecka, 2003). This might explain why vaccinated animals develop less severe clinical symptoms and lung lesions than non-vaccinated animals after challenge with *M. hyopneumoniae* (Meyns et al., 2006; Villarreal et al., 2011).

Production of pro-inflammatory and regulatory cytokines was also determined in vaccinated and non-vaccinated animals. Previous studies showed that vaccination can prevent the pathological effects of the inflammatory cytokines through the production of IL-10 (Conti et al., 2003). In the vaccinated pigs of our study, a significantly higher number of IL-10 secreting cells were detected in the bronchial lymph nodes (BLN) compared to the non-vaccinated pigs at 58 days of age. This may also play a role in the reduction of lung tissue damage after infection of vaccinated animals. It is well known that IL-10 has an anti-proliferative effect, so the induction of IL-10 secretion can reduce the macrophages influx in the bronchoalveolar lymphoid tissue. The reduction of macrophages in vaccinated pigs compared to non-vaccinated animals, after experimental infection with *M. hyopneumoniae*, has been described by Vranckx et al. (2012).

Seo et al. (2012) suggested that a higher induction of cell-mediated immunity against *M. hyopneumoniae* was associated with a lower severity of lung lesions induced by *M. hyopneumoniae*. To better understand the effect of vaccination on cellular immune responses, the production of IL-12 and IFN- γ was evaluated. Production of IL-12 by macrophages and B cells has been demonstrated to be important in the induction of a Th1 immune response (Yang et al., 2004). Furthermore, this cytokine stimulates IFN- γ production and enhances CD8⁺ cytotoxicity, both contributing to maintaining the cell mediated immunity (Park and Scott,

2001). Indeed, vaccinated pigs showed significantly higher amounts of IL-12 in spleen, BLN and lung tissues. At 58 days of age, the number of IFN- γ secreting cells was higher in the lungs of the vaccinated group. Significantly higher levels of IFN- γ were also found in the BAL fluid of the vaccinated group at 30 days of age, indicating that the vaccine also induced a local cellular immune response. Not only a local cellular immune response, but also IgG, IgM and IgA specific *M. hyopneumoniae* antibodies were detected in the BAL fluid of vaccinated animals. IgA was detected at all tested time points and may play an important role in protection by preventing the adhesion of mycoplasmas to the ciliated epithelial cells of the respiratory tract (Thacker et al., 2000).

Effects of vaccination on host immune responses induced by infection may play an important role in the protection against lung lesion development, as excessive levels of pro-inflammatory cytokines like IL-1 and IL-6 were observed in BAL fluid of *M. hyopneumoniae* infected pigs (Choi et al., 2006; Thacker et al., 2000) and have been associated with tissue damage and even death of the host (Asai et al., 1993; Okada et al., 2000). In this study, highest concentrations of IL-1 β and IL-6 were detected in the BAL fluid of the non-vaccinated group. This may suggest a regulatory effect of the vaccine in the release of these cytokines, avoiding tissue damage in the host.

Adjuvants may play an important role in the enhancement of the immunogenicity of an antigen and protection. The vaccine evaluated in the current study not only contained inactivated *M. hyopneumoniae* cells but also a complex adjuvant consisting of an acrylic acid polymer, carbopol 941® and squalane. Polyacrylic acid has been evaluated as adjuvant in veterinary vaccines against swine parvovirus (Gualandi et al., 1988), circovirus type 2 (Hoogland et al., 2006) and *Staphylococcus aureus* in sheep (Tollersrud et al., 2002). These reports showed that carbomers such as carbopol are not harmful in mammals and stimulate a more robust immune response than the antigen alone. In addition, it was demonstrated that carbopol drives an adaptive immune response with strong Th1-biased antibody production and T cell responses characterized by high levels of Th1 and Th2 cytokines when evaluated in mice (Krashias et al., 2010). Moreover, squalane was found to cause significant local immune stimulation and recruit and increase antigen uptake by dendritic cells that participate in antigen transport to draining lymph nodes (Calabro et al., 2011).

Very recently a study showed that vaccination pigs of less than one week of age with this commercial bacterin is effective in reducing lung lesions and performance losses due to *M. hyopneumoniae* infection (Wilson et al., 2012). However, further studies are still required to elucidate the mechanisms involved in induction of local antibodies and cellular immunity

after parenteral administration of this inactivated vaccine and its interaction with the protection against *M. hyopneumoniae* infection.

Development of new strategies for control of EP

The current control measures, including vaccination with the commercial vaccines, limit the economic damage. However, they provide only a partial protection, and do not constitute a sustainable control of EP (Thacker et al., 2000).

One of the disadvantages of bacterins is that they contain multiple antigens, and that some of these may even inhibit a protective immune response (Haesebrouck et al., 2004). The current bacterins are based on *in vitro* grown strains and do not contain antigens that are only expressed *in vivo*. Madsen et al. (2008) indeed observed differential protein expression between *in vivo* and *in vitro* grown strains. Furthermore, differences between circulating strains and strains included in commercial vaccines have been described which also might result in a lower efficacy of vaccination.

All licensed vaccines against EP should be applied parenterally, a route of immunization which, in general, is most effective for inducing systemic immune responses. However, as many respiratory pathogens, *M. hyopneumoniae* colonises mucosal surfaces, and the efficacy of the vaccines against this organism may be improved if both systemic and mucosal immune responses are induced. Many studies have demonstrated the efficiency of adhesin-based vaccines in blocking the interactions between the microbial adhesins and their host cell receptors (Wizemann et al., 1999). *M. hyopneumoniae* is primarily found on the mucosal surface of the trachea, bronchi, and bronchioles (Blanchard et al., 1992), and the adherence to the ciliated epithelium is a prerequisite for initiation of the infection. The mechanisms of adherence are complex and not fully understood. Zhang et al (1995) identified the P97 protein to be a ciliary adhesin. Many other potential adhesins have also been described (Burnett et al., 2006; Chen et al., 1998; Seymour et al., 2011; Seymour et al., 2012). However, some pure adhesins as well recombinant subunit vaccines containing only one antigen, elicit poor immune responses and need to be administered together with an immunostimulative carrier molecule or vector (Wizemann et al., 1999).

One strategy to induce mucosal immunity has been the use of bacterial enterotoxins such as cholera toxin (CT) from *Vibrio cholerae* and the related *Escherichia coli* heat labile enterotoxin (LT), which are highly immunogenic when delivered mucosally and parenterally and which can act as carriers to potentiate responses to non-related antigens (O'Hagan,

1998). Looking for a more complete protective response than the one generated by single component vaccines, a multi-component vaccine was developed. In the study presented in chapter 3.2, the aims were 1) to produce a recombinant chimeric protein composed of the c-terminal portion (R1) of the adhesin P97, P42 and NrdF antigens of *M. hyopneumoniae* fused to nontoxic *E. coli* heat-labile enterotoxin B subunit (LTB), 2) to evaluate the capacity of this chimeric protein to induce a specific immune response in intramuscularly and intranasally vaccinated mice, as well as in intramuscularly vaccinated pigs, and 3) to compare the effect of the fusion and co-administration of these proteins as well as the adjuvant effect of LTB.

Humoral and mucosal immune responses were characterized by the production of high levels of anti rP97R1, rP42 and rNrdF IgG in the sera and IgA antibodies in tracheobronchial lavage of vaccinated mice. Some of these antigens (P97R1 and NrdF) have previously been shown to induce specific immune responses in pigs too (Fagan et al., 2001; Okamba et al., 2010; Shimoji et al., 2003) and the heat shock protein P42 has been considered a good immunogen in several studies (Chen et al., 2003; Simionatto et al., 2012). In challenge experiments in pigs, it was shown that induction of antibodies against P97 or P97R1 and NrdF reduced the severity of lung lesions caused by *M. hyopneumoniae* infection (Fagan et al., 2001; Okamba et al., 2010; Shimoji et al., 2003). In addition, heat shock proteins as P42 have been shown to present multiple B-cell and T-cell epitopes and have also been used as an adjuvant to enhance cell-mediated immune responses in DNA vaccines (Chen et al., 2000).

Foreign antigens fused to recombinant LTB might constitute useful vaccines (da Hora et al., 2011), if these antigens indeed can be delivered across the mucosal epithelium to the underlying mucosa-associated lymphoid tissue. Then, they can stimulate the mucosal immune system by mediating the induction of antigen-specific mucosal IgA (Kim et al., 2011). LTB has been shown to be a potent mucosal and systemic adjuvant towards co-administered or fused unrelated antigens (Liu et al., 2011a; Zhou et al., 2009). It has already been shown that LTB can increase both T helper type 1 and 2 (Th1 and Th2) associated cytokine responses to LTB-fused antigens (Zhou et al., 2009). However, *E. coli* heat-labile toxin (LT) has also been shown to have negative side effects in humans, as both native and mutant LT (mLT) used as adjuvants were recently associated with the development of facial nerve paralysis following intranasal delivery in humans (Lewis et al., 2009). It is, therefore, evident that these toxin adjuvants exhibit safety concerns that will likely prevent their use in nasally delivered vaccines for humans.

Our results demonstrated that the presence of LTB, either co-administered or as part of the fusion proteins, resulted in significantly higher levels of IgG and IgA antibodies in

intranasally and intramuscularly immunized mice, although these levels differed between antigens, with higher antibody levels against the rP42. Even though no cytokine production was measured in the present study, it is commonly accepted that Th1 cells are preferentially involved in cell-mediated immunity, since they can 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 (Mosmann and Coffman, 1989). Intramuscular injection of the chimeric protein induced IgG1 and IgG2a. In the groups inoculated intranasally, significant differences were only found in the response against rP42, with higher levels in the group inoculated with the chimeric protein. These results indicate a mixed Th1 and Th2 response, mainly against the rP42.

The chimeric protein was also able to induce specific IgG antibodies in pigs, demonstrating the same profile as in mice. Significant differences were detected between pre-immunisation and post-immunisation serum antibody levels against all antigens comprising the chimeric protein, with higher levels of antibodies against rP42.

In conclusion, the study in chapter 3.2 described the design and expression of a chimeric recombinant protein comprising three *M. hyopneumoniae* antigens and the adjuvant LTB. The chimeric protein exhibited antigenic profiles similar to those of the original proteins, and the antigens included induced an immune response both in mice and in pigs. To our knowledge, this is the first multivalent subunit recombinant vaccine synthesized as a single molecule for *M. hyopneumoniae*. This approach may result in a formulation that could improve the protection against EP in pigs, and facilitate the manufacturing process, since more than one antigen can be produced in a single process. Further research will focus on the protective immunity of the vaccine against experimental *M. hyopneumoniae* infections in pigs.

Although the novel methodologies may show promising effects for the control of *M. hyopneumoniae* infections, very limited information is available on antigens of *M. hyopneumoniae* that are important for inducing protection. This is mainly due to the fact that the pathogen is very difficult to grow and manipulate *in vitro*. Antigens like P97, P97R1 and NrdF have been investigated in different vaccine formulations (Fagan et al., 2001; Ogawa et al., 2009; Okamba et al., 2010; Shimoji et al., 2003). Some of these formulations (P97 and NrdF) were able to induce specific immune responses in pigs but with only limited protection against pulmonary lesions caused by *M. hyopneumoniae* infection (Fagan et al., 2001; Shimoji et al., 2003). So, new and more efficient strategies are required to identify antigens that might be useful to the development of more effective vaccines.

Availability of the whole genome sequences of four *M. hyopneumoniae* strains (Liu et al., 2011b; Minion et al., 2004; Vasconcelos et al., 2005) has allowed the use of reverse vaccinology for development of recombinant subunit vaccines (Rappuoli, 2001). This approach may contribute to the identification of *M. hyopneumoniae* antigens that might be important for the development of new and more effective vaccines against EP. The main drivers for recombinant protein-based vaccine development are development of more potent, safer and better characterized vaccines, providing broader protection against multiple strains of a bacterium (Unnikrishnan et al., 2012).

In the study described in the chapter 3.3, recombinant antigens belonging to the group of transmembrane proteins of *M. hyopneumoniae* were evaluated as vaccine candidates. Transmembrane proteins play critical roles in cellular metabolism, participating in processes such as ion transport, nutrient uptake, signal transduction, and intercellular communication. The importance of these proteins is illustrated by the fact that more than half of all drug targets are transmembrane proteins. The analysis of the interactions of transmembrane proteins and their ligands is one of the most promising avenues for the discovery of new drug candidates as well as vaccine targets (Janulczyk and Rasmussen, 2001; Yoshino et al., 2010).

Six recombinant transmembrane proteins were cloned, expressed in *E. coli* and purified in sufficient amounts for use in further immunological analyses. This was not that easy as Mollicutes genomes have a high AT- content that reduces the expression levels of mycoplasmal proteins in heterologous expression systems, and there is poor intrinsic solubility and inherent toxicity of transmembrane proteins.

M. hyopneumoniae is a host specific pathogen which only infects pigs. Therefore, the efficacy of a vaccine against EP can only be determined in pigs, making the experiment laborious and expensive. Immunological characterization of each antigen is important in order to allow selection of the most qualified antigens for clinical trials in pigs. In chapter 3.3 of the present thesis, the immunogenicity of each recombinant protein was determined by inoculating purified antigens in mice and measuring the humoral immune response. All recombinant proteins evaluated were immunogenic for mice.

In addition, none of the recombinant proteins evaluated in this study reacted with the serum of the group of animals inoculated with the inactivated commercial vaccine. This indicates that these antigens are not present in the bacterin used in the experiment. This result is in agreement with a previous study, where a commercial vaccine did not stimulate the production of serum antibodies against the C-terminal portion of the adhesion P97 either, suggesting that the vaccine strain did not contain the P97 adhesin (Conceição et al., 2006).

The absence of this antigen playing a role in colonization, could compromise the efficacy of the vaccine, and might be one of the reasons why commercial vaccines only induce partial protection. Moreover, all recombinant proteins were recognized by serum from pigs naturally infected, indicating that they are expressed during infection, and may have potential to be used in serological diagnostic tests. However, a more detailed and efficient characterization needs to be performed before using these proteins as a tool for immunodiagnosis of EP.

The results of chapter 3.3 showed that six transmembrane proteins of *M. hyopneumoniae*, not previously characterized, are immunogenic and are expressed during infection in pigs. This suggests that these antigens may have potential to be included in a vaccine against EP, and that they might also be useful in diagnostic tests. Although the immune response of mice cannot be extrapolated to other species and no direct correlation has been found between serum antibody concentration and protection against *M. hyopneumoniae* challenge (Djordjevic et al., 1997), the results warrant further investigation in challenge experiments in pigs.

From the studies described in this thesis, it can be concluded that intramuscular vaccination with an adjuvanted bacterin is able to stimulate both a systemic and a local immune response. Also new approaches were designed for the development of vaccines against EP. However, further investigations are required to obtain better insights in the role of the host immune response in protection on the one hand and induction of lesions on the other hand. It was also concluded that a multi-component vaccine is a valuable strategy to develop new vaccines, since many *M. hyopneumoniae* antigens can be obtained in a single step production. It is clear that more studies are necessary to better understand the stability of the antigens in this single molecule and to investigate their efficacy in challenge experiments in pigs.

Future Perspectives

The investigated bacterin induced an immune response that might be important to inhibit the growth of *M. hyopneumoniae* and/or reduce the lesions caused by the infection. The immune responses of vaccinated animals following infection however are not fully understood, but infected pigs are unable to rapidly clear the infection. Further studies on bacteria–host interactions could allow selection of new protective antigens. Knowledge about the antigens of *M. hyopneumoniae* that are important for inducing protection is limited and more information on these antigens is necessary, as this is an essential step for the development of more effective vaccination and control strategies.

The identification of new antigens has been facilitated by the fact that the genome of four different *M. hyopneumoniae* isolates has been sequenced and recombinant antigens evaluated. The antigens identified in this thesis were immunogenic in mice, are a first step in the vaccine development. They need to be tested in pigs for their possible protective immunity. In addition, from an immunological point of view, further research should focus on induction of immunity at the mucosal level. As *M. hyopneumoniae* organisms adhere to the ciliated epithelial cells of the trachea, bronchi and bronchioles, local IgA responses may be important for protection.

Since even with the best antigen identification production system, vaccines might not reach their full potential unless they are formulated and delivered properly. Therefore, in addition to identifying new antigens potentially inducing mucosal immunity, research should also focus on other factors such as the formulation and delivery of the antigens, and the adjuvants leading to an appropriate and long-lasting protective immunity. For example, heterologous prime – boost immunisation strategies comprising priming with a DNA vaccine followed by boosting with recombinant proteins may be useful to enhance the immune response. A combination of the mentioned factors may facilitate the design and production of more potent and safer vaccines against EP, and vaccines that are also easy to administer.

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SUMMARY

Summary

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is known to be the primary causative agent of enzootic pneumonia in pigs. This disease leads to a chronic dry cough and slight hyperthermia, reduces the pig's average daily weight gain and feed conversion efficiency, causes low mortality but high morbidity. The infection of *M. hyopneumoniae* may also increase the susceptibility of pigs to other pathogens such as *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus, and may cause more serious pneumonia once complicated with infections of other respiratory pathogens. So it is a key pathogenic microorganism in porcine enzootic pneumonia (EP) and the porcine respiratory disease complex (PRDC). Both diseases represent serious health concerns worldwide in swine populations, and cause significant economic losses in pig production.

M. hyopneumoniae infection can be controlled by optimizing management practices and housing conditions, the use of antibiotics and vaccination. Commercial vaccines consist of adjuvanted inactivated whole cell preparations, and are frequently used worldwide in countries with an intensive pig production. The currently available vaccines are beneficial from an economic point of view, but they do not provide a sustainable control of the disease. They cannot prevent colonization of *M. hyopneumoniae* in the respiratory tract, and do not significantly reduce the transmission of the pathogen. Also, little is known about the exact mechanisms of the partial protection they induce.

The general aims of this thesis were to assess the mode of the action of an existing commercial vaccine on the one hand and to develop and evaluate new recombinant vaccines against *M. hyopneumoniae* on the other hand, looking for more effective strategies for disease control. For development of the recombinant vaccines, two types of antigens were considered. One vaccine was based on proteins known to play a role in the adhesion and that were able to induce a partial protection against *M. hyopneumoniae* infection when evaluated individually. The other antigens were a group of transmembrane proteins (not previously characterized). The identification of these novel antigens might contribute to the development of improved vaccines.

The specific objectives were:

1. To analyze the systemic as well as the local mucosal immune response induced by intramuscular administration of a commercial *M. hyopneumoniae* vaccine in pigs.

2. To produce a recombinant chimeric protein composed of the P97R1, P42 and NrdF antigens of *M. hyopneumoniae* fused to *Escherichia coli* heat labile enterotoxin B subunit (LTB) and to evaluate the capacity of this chimeric protein to induce a specific immune response in mice and pigs.
3. To identify, clone and characterize transmembrane proteins from *M. hyopneumoniae* which may be useful in vaccination against enzootic pneumonia.

In chapter 1, a review of the literature is given, with emphasis on vaccination strategies, including the use of reverse vaccinology approaches. The aims of the thesis are shown in chapter 2. The three experimental studies are presented in chapter 3.

In the first study (Chapter 3.1), the mechanisms were investigated by which a commercial *M. hyopneumoniae* bacterin, applied once intramuscularly in piglets of 7 days old, can induce a systemic as well as a local mucosal immune response against EP. Pigs were euthanized and necropsied at 30, 36 and 58 days of age. Immunological assays were performed on blood, bronchoalveolar lavage (BAL) fluid, lung tissue, bronchial lymph nodes (BLN) and spleen of vaccinated and non-vaccinated pigs. Serum and BAL fluid were tested for the presence of antibodies by ELISA. Mononuclear cells from the peripheral blood and tissues were isolated to quantify the T cell subsets by flow cytometry, and cytokine production by ELISpot and ELISA. Antibodies against *M. hyopneumoniae* were detected in serum of most vaccinated pigs at 30 days of age. *M. hyopneumoniae* specific IgG, IgM and IgA were detected in BAL fluid from vaccinated animals, but not from control animals. Significantly higher numbers of IL-12 secreting cells were observed in the lung at day 58 in the vaccinated than in the non-vaccinated group ($p < 0.05$). The number of IL-10 secreting cells from BLN was also higher in the vaccinated group at day 58 ($p < 0.05$). After restimulation *in vitro*, lymphocytes from BLN and lungs secreted significantly higher levels of IL-12 in the vaccinated group at day 58. These results show that the vaccine induced both systemic and mucosal cellular and humoral immune responses.

In the study described in the chapter 3.2, a multi-antigen chimera with three antigens of *M. hyopneumoniae* (R1, P42 and NrdF) and the mucosal adjuvant LTB was constructed and its antigenic and immunogenic properties in mice and pigs were evaluated. In addition, a comparison between fusion and co-administration of these proteins was performed. The chimeric protein was constructed and expressed in *E. coli*. Antibodies against each subunit recognized the chimeric protein, showing its antigenicity. Intranasal and intramuscular immunization of mice with the chimeric protein significantly increased the levels of mice IgG

and IgA, in serum and tracheobronchial lavages, respectively. Mice immunized with chimeric protein, showed levels of IgG and IgA antibodies against rP42 significantly higher in both intramuscular and intranasal immunization ($p < 0.005$), when compared with pre-immune serum. Pigs immunized with the chimeric protein developed an immune response against all *M. hyopneumoniae* antigens present in the fusion with statistically significant difference ($p < 0.05$) compared to the pre-immune serum. Highest immune responses were detected in fused and co-administered antigens with adjuvant, proving the adjuvant effect of rLTB. However, better results were shown by the chimeric protein, highlighting the ability of fused antigens to stimulate an immune response. This multi-antigen is a promising vaccine candidate that may help to control *M. hyopneumoniae* infection.

The availability of the whole genome sequences of four *M. hyopneumoniae* strains has allowed to use reverse vaccinology for development of recombinant subunit vaccines. This approach may contribute to the identification of *M. hyopneumoniae* antigens that might be important for the development of new and more effective vaccines against EP. In the third study (Chapter 3.3), 17 genes coding for transmembrane proteins from *M. hyopneumoniae* were cloned, among which six were successfully expressed in *E. coli* and their immunogenic and antigenic properties were investigated. All proteins were immunogenic in mice and sera from naturally infected pigs reacted with the recombinant proteins, suggesting that they are expressed during infection. These antigens may contribute to the development of new recombinant vaccines and diagnostic tests for EP.

From the studies described in this thesis, it can be concluded that intramuscular vaccination with an adjuvanted bacterin is able to stimulate both systemic and local immune responses. New approaches were designed for the development of vaccines against EP. It was also concluded that a multi-component vaccine is a valuable approach to develop new vaccines, since many *M. hyopneumoniae* antigens can be obtained in a single step production. It is clear that more studies are necessary in order to better understand the stability of the antigens in this single molecule and their efficacy in challenge experiments in pigs will also have to be determined. Further investigations are required in order to obtain better insights in the role of the host immune response in protection on the one hand and induction of lesions on the other hand.

SAMENVATTING

Samenvatting

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is het primaire agens dat enzoötische pneumonie bij varkens veroorzaakt. Deze ziekte gaat gepaard met een chronische, droge hoest, lichte koorts, een verminderde dagelijkse groei en hogere voederconversie, hoge morbiditeit en meestal een lage mortaliteit. Een infectie met *M. hyopneumoniae* verhoogt de gevoeligheid van varkens voor infecties met andere pathogenen zoals *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, porcien reproductief en respiratoir syndroom virus (PRRSV) en porcien influenzavirussen. Een secundaire besmetting met deze respiratoire pathogenen na een infectie met *M. hyopneumoniae* resulteert in meer uitgesproken symptomen en letsels. Men spreekt dan over enzoötische pneumonie (EP) of het porcien respiratoir ziekte complex. In dit laatste geval gaat het over infecties met *M. hyopneumoniae* in combinatie met virale pathogenen en het ziektebeeld is meer acuut in vergelijking met EP. Beide aandoeningen zorgen wereldwijd voor grote gezondheidsproblemen bij varkens en belangrijke economische verliezen voor de varkenssector.

M. hyopneumoniae infecties kunnen onder controle gehouden worden door de bedrijfsvoering en de huisvesting op het bedrijf te optimaliseren, door het gebruik van antibiotica en het toepassen van vaccinatie. De huidige commerciële vaccins bevatten geïnactiveerde *M. hyopneumoniae* bacteriën en een adjuvans. Vaccinatie wordt veelvuldig en wereldwijd aangewend in landen met een intensieve varkenshouderij. Deze huidige vaccins zijn effectief vanuit economisch oogpunt, maar ze zijn niet in staat om de ziekte volledig onder controle te houden. De kolonisatie van *M. hyopneumoniae* ter hoogte van het ademhalingsstelsel wordt niet verhinderd en de verspreiding van het agens wordt niet significant verminderd door vaccinatie. Toen dit doctoraatsonderzoek opgestart werd, was het onvoldoende gekend welke immunomechanismen een rol spelen bij het induceren van de partiële bescherming na vaccinatie met commerciële vaccins.

De algemene doelstellingen van deze doctoraatssthesis waren enerzijds het nagaan van het werkingsmechanisme van een bestaand commercieel vaccin en anderzijds het ontwikkelen en evalueren van nieuwe vaccins op basis van recombinant aangemaakte eiwitten van *M. hyopneumoniae*, om op die manier te komen tot meer effectieve controlestrategieën. Voor de ontwikkeling van de nieuwe vaccins werd gebruik gemaakt van twee types antigenen. Een eerste vaccin was gebaseerd op drie eiwitten die een rol spelen in de adhesie van de kiem ter hoogte van het ademhalingsstelsel. In de tweede plaats werd het immunogeen vermogen

nagegaan van recombinant tot expressie gebrachte transmembranaire proteïnen die voordien nog niet gekarakteriseerd werden.

De specifieke doelstellingen van deze doctoraatsthesis waren:

1. Het bestuderen van de systemische en mucosale immuunrespons bij varkens die intramusculair gevaccineerd werden met een commercieel *M. hyopneumoniae*-vaccin.
2. Het produceren van een recombinant chimeer eiwit bestaande uit drie adhesines van *M. hyopneumoniae* (P97R1, P42 en NrdF) gefuseerd met het B fragment van het thermolabiele enterotoxine van enterotoxigene *Escherichia coli* (LTB) en het nagaan van de capaciteit van dit chimeer eiwit om een specifieke immuunrespons te induceren bij muizen en varkens.
3. Het identificeren, klonen en karakteriseren van transmembranaire proteïnen van *M. hyopneumoniae* die eventueel bruikbaar kunnen zijn voor het ontwikkelen van vaccins tegen enzoötische pneumonie.

In hoofdstuk 1 wordt een literatuuroverzicht gegeven in verband met *M. hyopneumoniae*, met nadruk op vaccinatie, inclusief het principe van “reverse vaccinology”. De doelstellingen van deze doctoraatsthesis worden gegeven in hoofdstuk 2 en de drie uitgevoerde experimentele studies worden besproken in hoofdstuk 3. Tenslotte worden in de algemene discussie (hoofdstuk 4) de voornaamste bevindingen bediscussieerd en worden suggesties gegeven voor verder onderzoek.

In de eerste experimentele studie (hoofdstuk 3.1) werd de systemische en mucosale immuun respons bestudeerd bij biggen die op de leeftijd van 7 dagen eenmalig intramusculair gevaccineerd werden met een commercieel *M. hyopneumoniae* bacterin. De dieren werden geëuthanaseerd en daarna gelijkgeschouwd op de leeftijd van 30, 36 en 58 dagen. Immunologische testen werden uitgevoerd op het bloed, longspoelvocht, de long, bronchiale lymfeknopen en de milt van gevaccineerde dieren en niet gevaccineerde controle varkens. Het serum en het longspoelvocht werden getest op de aanwezigheid van antistoffen door middel van ELISA. Monomorfonucleaire cellen werden uit het perifeer bloed en de weefsels geïsoleerd om de aanwezigheid van T-cellen na te gaan door middel van flowcytometrie. Tevens werd door middel van ELISpot en ELISA de cytokineproductie door deze cellen onderzocht. Antistoffen tegen *M. hyopneumoniae* werden teruggevonden in het serum van de meeste gevaccineerde varkens op 30 dagen leeftijd. *M. hyopneumoniae* specifieke IgG, IgM en IgA konden gedetecteerd worden in het longspoelvocht van gevaccineerde dieren, maar niet bij de controledieren. Significant hogere aantallen IL-12-secreterende cellen werden bij

de gevaccineerde dieren aangetroffen in de longen op 58 dagen leeftijd, in vergelijking met de niet-gevaccineerde dieren ($p < 0.005$). Het aantal IL-10-secreterende cellen in de bronchiale lymfeknopen was ook hoger bij de gevaccineerde groep op 58 dagen leeftijd ($p < 0.05$). Na herstimulatie van de lymfocyten uit de bronchiale lymfeknopen en de longen, werden er significant hogere hoeveelheden IL-12 gesecreteerd bij de gevaccineerde dieren van 58 dagen oud. Deze resultaten tonen aan dat het vaccin zowel een systemische als een mucosale cellulaire en humorale immuunrespons induceert.

In de studie beschreven in hoofdstuk 3.2 werd een multi-antigen chimeer met drie adhesines van *M. hyopneumoniae* (R1, P42 en NrdF) en het LTB geconstrueerd. Het LTB fungeerde als mucosaal adjuvans. De antigenen en immunogene eigenschappen werden zowel bij muizen als bij varkens onderzocht. Bovendien werd het gelijktijdig toedienen van de afzonderlijke adhesines vergeleken met het toedienen als multi-antigen chimeer. Het chimeer-eiwit werd geconstrueerd en tot expressie gebracht in *E. coli*. Antistoffen gericht tegen elke subunit herkenden het chimeer-eiwit, wat de antigenen eigenschappen van dit eiwit aantoont. Intranasale en intramusculaire immunisatie van muizen met het chimeer-eiwit deed het aantal IgG en IgA-antistoffen in respectievelijk het serum en longspoelvocht significant toenemen. Muizen die geïmmuniseerd werden met het chimeer-eiwit vertoonden significant hogere concentraties IgG- en IgA-antistoffen gericht tegen rP42 dan in het pre-immunisatieserum ($p < 0,05$), zowel na intramusculaire als na intranasale immunisatie. Varkens die geïmmuniseerd werden met het chimeer-eiwit bouwden antistoffen op tegen alle *M. hyopneumoniae* antigenen aanwezig in het chimeer-eiwit. Dieren die geïmmuniseerd werden met het multi-antigen chimeer vertoonden de hoogste immuunrespons. Dit kan er op duiden dat een dergelijk multi-antigen chimeer een bruikbaar vaccin zou kunnen zijn. Verdere studies waarbij gevaccineerde varkens experimenteel besmet worden met *M. hyopneumoniae* zijn evenwel noodzakelijk om dit te bevestigen.

De volledige genoom-sequenties van vier *M. hyopneumoniae*-stammen zijn beschikbaar. Dit maakt het mogelijk om “reverse vaccinology” toe te passen voor de ontwikkeling van recombinante subunit vaccins. In de derde studie (hoofdstuk 3.3) werden 17 genen die coderen voor transmembranaire proteïnen van *M. hyopneumoniae*, gekloond. Van deze 17 genen werden er zes succesvol tot expressie gebracht in *E. coli*. De immunogene en antigenen eigenschappen van de aldus tot expressie gebrachte eiwitten werden onderzocht. Alle eiwitten vertoonden immunogene eigenschappen bij muizen en serum van natuurlijk geïnfecteerde varkens reageerde met de recombinante proteïnen. Dit suggereert dat deze proteïnen tot expressie worden gebracht tijdens een *M. hyopneumoniae* infectie. Deze

antigenen kunnen een bijdrage leveren tot de ontwikkeling van nieuwe recombinante vaccins tegen EP en diagnostische testen voor de detectie van deze ziekte.

Als conclusie kan gesteld worden dat intramusculaire vaccinatie met een commercieel beschikbaar geïnactiveerd vaccin op basis van *M. hyopneumoniae* kiemen zowel een systemische als lokale immuunrespons uitlokt. Er werd ook aangetoond dat een chimeer vaccin bestaande uit een fusieproteïne van drie *M. hyopneumoniae* adhesines en het mucosaal adjuvans LTB, een immuun respons induceert tegenover deze drie adhesines. Dit kan er op duiden dat deze benadering nuttig kan zijn voor ontwikkeling van nieuwe vaccins. Ook recombinant tot expressie gebrachte transmembranaire proteïnen van *M. hyopneumoniae* waren immunogeen. De stabiliteit van chimere vaccins dient nog nader onderzocht te worden en hun doeltreffendheid moet nagegaan worden door middel van infectieproeven bij varkens. Verder onderzoek is ook nodig om betere inzichten te verkrijgen in de rol die de immuunrespons van de gastheer speelt in het verkrijgen van bescherming enerzijds en het induceren van letsels anderzijds.



Curriculum Vitae

Silvana Beutinger Marchioro was born on June 28th 1983, in Ijuí, Rio Grande do Sul, Brazil. She finished her secondary studies in 2000 in the Evangelical College Augusto Pestana. She graduated in Veterinary Medicine at the Federal University of Santa Maria, in January 2007.

Her interest in research led her to start a master thesis in March of 2007 in the Biotechnology program of Federal University of Pelotas, obtaining her Master in Sciences degree in October of 2008. It was during this period that she started her research on *Mycoplasma hyopneumoniae*. In November 2008, she started a PhD program in the same Faculty, working on the same subject. In 2011, she was awarded with a scholarship from the CAPES Foundation, an agency under the Ministry of Education of Brazil, in order to conduct research on *Mycoplasma hyopneumoniae* as a Visiting Graduate Student at the Faculty of Veterinary Medicine at Ghent University (Belgium), for a period of one year. In 2012, she worked on a project for a joint PhD of the Special Research Fund (BOF) of Ghent University (scholarship's code 01SF0611). The research at Ghent University took place at the Department of Pathology, Bacteriology and Avian diseases and at the Department of Reproduction, Obstetrics and Herd Health.

Silvana is author and co-author of several studies published in international peer reviewed journals. Her experimental work has been presented in different national and international congresses.

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