**There is nothing impossible to him who will try.**

**Alexander the Great (born in Pella, 356 BC)**

FACULTY OF VETERINARY MEDICINE
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# Different vaccination protocols with bacterins as a means to control *Mycoplasma hyopneumoniae* infections in peri-weaned and fattening pigs

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| *A. pleuropneumoniae* | *Actinobacillus pleuropneumoniae* |
| ANOVA | Analysis of variance |
| ADG | Average daily weight gain |
| *B. bronchiseptica* | *Bordetella bronchiseptica* |
| BALF | Broncho-alveolar lavage fluid |
| CCU | Color changing units |
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| F1 | First fattening unit |
| F2 | Second fattening unit 2 |
| *H. parasuis* | *Haemophilus parasuis* |
| IF | Immunofluorescence |
| Ig | Immunoglobulins |
| IHC | Immunohistochemistry |
| i.m. | Intramuscular |
| LLS | Lung lesion score |
| *M. flocculare* | *Mycoplasma flocculare* |
| *M. hyopneumoniae* | *Mycoplasma hyopneumoniae* |
| *M. hyorhinis* | *Mycoplasma hyorhinis* |
| *nPCR* | nested Polymerase chain reaction |
| NV | Non-vaccinated |
| OD | Optical density |
| *P. multocida* | *Pasteurela multocida* |
| PCR | Polymerase chain reaction |
| PEP | Porcine enzootic pneumonia |
| PCV-2 | Porcine circovirus type 2 |
| PRRSV | Porcine reproductive and respiratory syndrome virus |
| PRDC | Porcine respiratory disease complex |
| PRV | Pseudorabies virus |
| qPCR | quantitative real-time Polymerase chain reaction |
| RDS | Respiratory disease score |
| SD | Standard deviation |
| *S. suis* | *Streptococcus suis* |
| SIV | Swine influenza virus |
| TBS | Tracheobronchial swabs |
| *T. pyogenes* | *Trueperella pyogenes* |
| IP values | Inhibition percentage values |
| USA | United States of America |
| V | Vaccinated |
| V1 | Vaccinated three days before weaning |
| V2 | Vaccinated at the day of weaning |

# General introduction

## REVIEW OF THE LITERATURE

Worldwide, respiratory diseases are considered as some of the most serious problems faced by the swine industry. *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is a bacterial pathogen commonly present in almost all swine-producing areas around the world (Thacker and Minion, 2012). It is the causative agent of porcine enzootic pneumonia (PEP) and one of the primary pathogens involved in the porcine respiratory disease complex (PRDC). PEP is a chronic respiratory disease that results from infection of *M. hyopneumoniae* and other secondary bacteria. The disease induces major economic losses to the swine industry, due to pig growth retardation, higher feed conversion ratios and increased antimicrobial use (Maes *et al*., 2008). PRDC is a broader term used to describe the polymicrobial etiology of respiratory disease in modern commercial production conditions, where apart from several bacteria *M. hyopneumoniae* can additionally interact with viruses, and possibly parasites (Van Alstine, 2012).

Within herds, *M. hyopneumoniae* is transmitted vertically from the sows to their piglets or horizontally between pigs sharing the same airspace. Several factors can influence its transmission, namely the virulence of the strains circulating, the type of production system, the housing and management conditions and also, the infection status of the breeding sows (Maes *et al*., 2017).

It is generally acknowledged that in many herds the pigs are already infected during the peri-weaning period (Garza-Moreno *et al*., 2018). In these herds, the infection status of the sows around farrowing can be an important risk factor for colonization of their piglets (Calsamiglia and Pijoan, 2000). It has been shown that the level of piglet colonization with *M. hyopneumoniae* at weaning could be a possible predictor of the severity of PEP-induced clinical disease and PEP-like lung lesions at slaughter (Fano *et al*., 2007; Sibila *et al*., 2007a).

Under field conditions, an accurate and conclusive diagnosis of *M. hyopneumoniae* infections needs the combination of clinical examination and macroscopic lung lesion evaluation, together with laboratory techniques that demonstrate the presence of the pathogen, preferably in the lung lesions (Thacker, 2004; Thacker and Minion, 2012). Diagnostics can also be used to determine the dynamics of infection across the different age groups and production sites. In that way, the herd veterinarian can decide better on the most appropriate control strategies to be applied.

Vaccination with commercial bacterins is one of the most common ways to control *M. hyopneumoniae* infections, together with the use of antimicrobials, and the optimization of the management practices and microclimate conditions in the production units (Maes *et al*., 2017). This review aims to discuss the current knowledge on *M. hyopneumoniae* infections and PEP, with emphasis on the epidemiology, diagnosis and control of the disease.

## 1.1. CHARACTERISTICS OF *MYCOPLASMA HYOPNEUMONIAE*

Mycoplasmata are taxonomically specified as members of the class Mollicutes (Razin, 2006). The bacteria of this class are characterized by their diminutive size and the absence of a cell wall. Bacteria belonging to the *Mycoplasma* genus have a small genome (580-1350 kbp) and as a result, they undergo limited metabolism and possess limited biosynthetic pathways (Razin *et al*., 1998; Simionatto *et al*., 2013). This means that they need to obtain the necessary metabolites from their direct environment (Thacker and Minion, 2012).

*M. hyopneumoniae* organisms are coccoid or coccobacillary to short filamentous bacteria, with their size ranging between 0.3 to 0.8 μm. *M. hyopneumoniae* is a very fastidious organism to culture in the laboratory and extremely difficult to isolate from clinical samples. For that reason, *in vitro* cultivation requires a specialized medium supplemented with serum. So far, the medium developed by Friis has been the most commonly used liquid medium for the culture and isolation of the organism (Friis, 1975; Simionatto *et al*., 2013). In the meantime though, several new types of media that constitute an improved version of the Friis medium have been developed (Hwang *et al*., 2010; Cook *et al*., 2016). Nevertheless, *M. hyopneumoniae* culture is so laborious and time-consuming that it can take up to two months in order to obtain a purified isolate. Additionally, contamination by *Mycoplasma hyorhinis* (*M. hyorhinis*) and *Mycoplasma flocculare* (*M. flocculare*) often complicates the isolation of the organism. If present, those commensal respiratory bacteria can easily overgrow *M. hyopneumoniae*, thus rendering isolation procedures to be unsuccessful (Thacker and Minion, 2012).

An experimental study conducted by Vicca *et al*. (2003) demonstrated significant differences in virulence between different field isolates of *M. hyopneumoniae* isolates. To date, differences in virulence among isolates have not been predominantly attributed to presence or absence of specific virulence genes. In contrast, virulence has been rather implied to be influenced by

differences in the expression level of such virulence genes (Li *et al*., 2009; Pinto *et al*., 2009; Berry *et al*., 2017).

## 1.2. PATHOGENESIS OF *MYCOPLASMA HYOPNEUMONIAE* INFECTIONS

*M. hyopneumoniae* is a host specific pathogen that only infects wild boars and domestic pigs. The pathogenesis and especially virulence factors of *M. hyopneumoniae* are not yet fully understood. Adhesion of the organism to the ciliated epithelium of the respiratory tract leads to disruption of the mucociliary clearance system (Blanchard *et al*., 1992; Sarradell *et al*., 2003). This process is a prerequisite for the initiation of disease (Razin *et al*., 1998). Additionally, the organism suppresses and modulates the innate and adaptive respiratory immune responses (Simionatto *et al*., 2013). All the above are important steps of the pathogenesis, leading to the prolonged persistence of the pathogen in the respiratory tract and damage of the pulmonary tissue.

### 1.2.1. Adherence of the pathogen to the respiratory tract

Upon inhalation, *M. hyopneumoniae* attaches to the ciliated epithelial cells of the trachea, the bronchi and the bronchioles, but does not penetrate into the lung parenchyma (Figure 1; Blanchard *et al*., 1992). The exact method of attachment to the cilia has not yet been fully elucidated, nevertheless several *M. hyopneumoniae* surface proteins involving in this process have been identified (Zhang *et al*., 1995; Pinto *et al*., 2007; Wilton *et al*., 2009). These are Mhp182 (P102), Mhp183 (P97), Mhp 493 (P159), Mhp 494 (P216), Mhp 683 (P135), Mhp 271, Mhp 107 and Mhp 108 (P116). These adhesins are post-translationally processed and cleaved at multiple sites generating a repertoire of cleavage fragments that remain attached on the extracellular surface of *M. hyopneumoniae*. In that way, the pathogen acquires antigenic and functional variation, enabling it to regulate adhesion to the host tissues and to evade clearance by the immune system (Minion *et al*., 2000; Seymour *et al*., 2010).

Upon adherence to the ciliated respiratory epithelia, *M. hyopneumoniae* causes degenerative changes (Blanchard *et al*., 1992). These are ciliostasis, clumping and loss of cilia as well as destruction of the epithelial cells. As a result, the mucosal clearance system of the respiratory tract

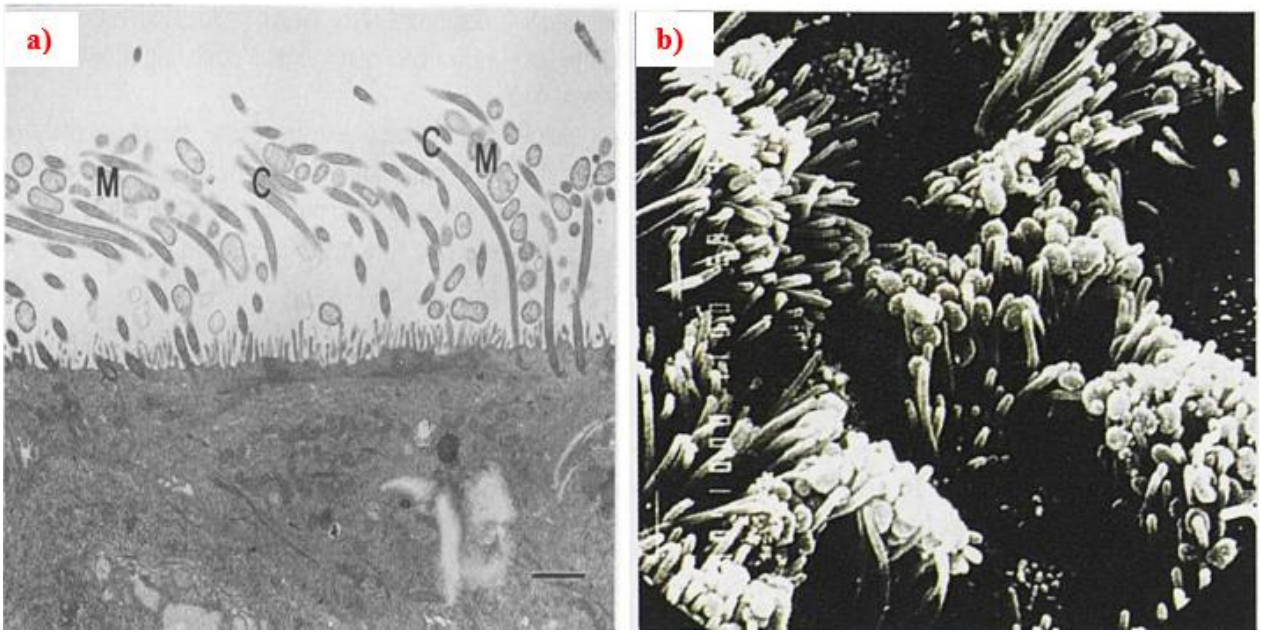



**Figure 1**. Scanning electron microscopy pictures showing the adherence of *M. hyopneumoniae* to the epithelial cilia lining of the trachea in challenge infected pigs: **a)** picture obtained from the study of DeBey and Ross (1994), where *M. hyopneumoniae* cells (M) are attached to the cilia (C) as well as to the tips of the microvilli, and **b)** picture obtained from Kobisch and Friis (1996), where *M. hyopneumoniae* cells are attached to the tops of the cilia.

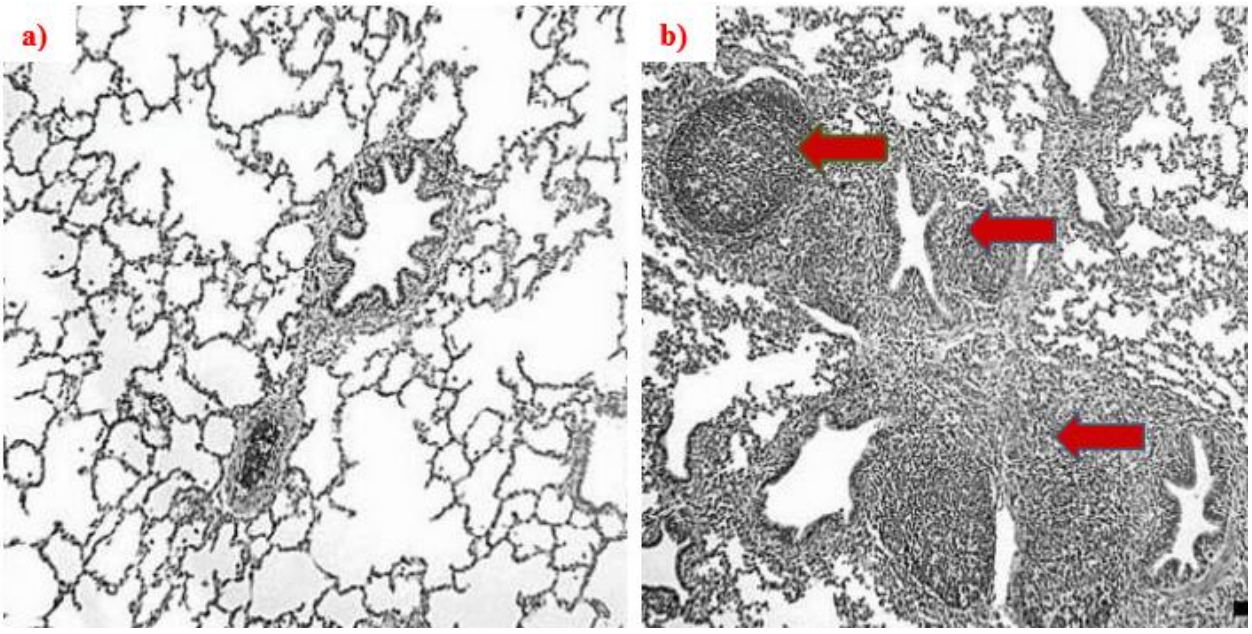



**Figure 2**. Histological examination of the lung tissue of a clinically healthy (control) pig **(a)**, together with a lung tissue section from a pig challenge infected with *M. hyopneumoniae* **(b)** from the study of Opriessnig *et al*. (2004). In the infected pig there is peribronchiolar and perivascular lymphocytic hyperplasia and infiltration of macrophages and neutrophils within airway lumens (arrows).

becomes ineffective (Thacker and Minion, 2012). This leads to the further multiplication and spread of *M. hyopneumoniae* in the respiratory tract and also, to a significant reduction in the mucociliary clearance of debris and other respiratory pathogens (e.g. *Bordetella bronchiseptica*, *Pasteurella multocida*, *Streptococcus suis*). The loss of cilia is more apparent in the airways of the cranioventral lobes of the lungs. This distribution is associated with the predominant location of macroscopic lesions caused by PEP (Mebus and Underdahl, 1977).

The mechanism behind cilia damage is not fully understood, but it has been suggested that *M. hyopneumoniae* causes an increased inflow of $Ca^{2+}$ in the respiratory epithelial cells (Park *et al*., 2002). Another possible mechanism by which *M. hyopneumoniae* has been proposed to cause degeneration of the ciliated epithelial cells is through the production of hydrogen peroxide ($H_2O_2$), which is a by-product of glycerol metabolism by glycerolphosphate oxidase (Simionatto *et al*., 2013; Ferrarini *et al*., 2016). A highly conserved homologous gene has been found in its genome (named *glpD*), similar to the genes that code for glycerolphosphate oxidase (i.e. the enzyme that metabolizes glycerol derived from phospholipids of human or animals hosts to $H_2O_2$) in other mycoplasmas (Ferrarini *et al*., 2016). Nevertheless, functional assays have failed to detect $H_2O_2$ production by several *M. hyopneumoniae* strains, hence this pathogenesis mechanism remains unresolved.

### 1.2.2. Interaction with the immune system

It has been shown that *M. hyopneumoniae* has a modulating effect on the immune system. Immediately after infection, there is an infiltration of macrophages and lymphocytes in the perivascular, peribronchial and peribronchiolar areas and alveolar septa, as well as lymphoid hyperplasia (Figure 2; Baskerville, 1972; Sarradell *et al*., 2003). These immunopathological events result in a prolonged pulmonary inflammatory response that plays an important role in the development of lung lesions and the exacerbation of *Mycoplasma*-induced disease.

***Innate immune response***

Upon infection, *M. hyopneumoniae* triggers the secretion of pro-inflammatory cytokines (IL-1, IL-6, IL-8 and TNF-α) by the pulmonary alveolar macrophages (Asai *et al*., 1993, Rodríguez *et al*., 2004). These cytokines are important mediators in the initiation of inflammation in the lung parenchyma. Moreover, different *M. hyopneumoniae* strains may induce the production of cytokines to a different degree. Meyns *et al*. (2007) showed that at days five and 28, and at days 10 and 15 post-infection, respectively, higher concentrations of IL-1β and TNF-α were detected in the broncho-alveolar lavage fluid (BALF) of pigs experimentally infected with a highly virulent strain compared to the pigs infected with a low virulent strain. Additionally, during the early stages of infection, *M. hyopneumoniae* has been shown to inhibit macrophage-mediated phagocytosis (Caruso and Ross, 1990). This immunosuppressive effect does not only reduce the clearance of *M. hyopneumoniae*, but also of other respiratory pathogens that are mainly secondary bacteria involved in the development of PEP.

***Adaptive immune response***

The role of the T-cells in the pathogenesis of *M. hyopneumoniae* infections has been highlighted by Tajima *et al*., (1984) who found that suppression of the T-cell response achieved by thymectomy prior to experimental infection resulted in decreased severity of lung lesions compared to immunocompetent infected pigs. However, *M. hyopneumoniae* was isolated from the spleen of a thymectomized pig (Tajima *et al*., 1984). These results suggested that a T-cell-dependent mechanism may be important in the development of pneumonia, but is also important in preventing systemic spread of *M. hyopneumoniae*. Additionally, helper T-cells are the most numerous subset in the lymphoid infiltration of the peribronchiolar and perivascular areas of the lung in infected pigs, although cytotoxic T-cells are also present (Okada *et al*., 2000; Sarradell *et al*., 2003).

Cytokines IL-2 and IL-4 are consistently expressed in various mononuclear cells (lymphocytes and macrophages) of the broncho-alveolar exudates of challenge infected pigs after the first week post-infection (Lorenzo *et al*., 2006), and have been incriminated in the characteristic peribronchiolar lymphoid hyperplasia associated with PEP (Rodríguez *et al*., 2004).

During the evolution of *Mycoplasma hyopneumoniae*-induced pneumonia, there is a suppression of the IL-10 and the *M. hyopneumoniae*-specific and non-specific IFN-ɣ cytokine responses (Muneta *et al*., 2008). IL-10 has an anti-macrophage activity and its suppression may lead to over-activation of macrophages, which exacerbates lung tissue damage (Muneta *et al*., 2008). IL-10 has been associated with clinical protection and reduced lung lesion severity (Moore *et al*., 1993; Morrison *et al*., 2000). Indeed, under experimental conditions induction of IL-10 secretion by vaccination resulted in a lower influx of macrophages in the bronchial lymph node tissue of the vaccinated pigs when compared to the non-vaccinated pigs (Vranckx *et al*., 2012a). This could explain why the infiltration of macrophages after infection with *M. hyopneumoniae* is reduced by vaccination (Vranckx *et al*., 2012a).

Thacker *et al*. (2000a) evaluated the cell-mediated immune response induced by vaccination or experimental challenge infection with *M. hyopneumoniae*. In this context, ELISPOT assays to identify IFN-γ secreting cells were performed. They discovered that pigs vaccinated against and challenged with *M. hyopneumoniae* had the greatest number of IFN-γ secreting cells on day seven post-infection. They suggested that T helper (type 1) and natural killer cells secreting IFN-γ may be important in mediating a protective immune response against *Mycoplasma hyopneumoniae*-induced pneumonia. IFN-γ cytokine responses also play an important role in the clearance and protection against viruses, and their suppression by *M. hyopneumoniae* may enhance secondary viral infections such as porcine reproductive and respiratory syndrome virus (PRRSV; Thacker *et al*., 1999) and swine influenza virus (SIV; Yazawa *et al*., 2004).

In addition to the aforementioned, Kishima and Ross (1985) demonstrated that cell membranes of *M. hyopneumoniae* reduced the responsiveness of lymphocytes to the non-specific T-cell mitogen phytohemagluttanin, and suggested that *M. hyopneumoniae* has a general immunosuppressive effect on the cell-mediated immune responses.

After infection with *M. hyopneumoniae*, the induction of systemic humoral immune response is considered to be a slow procedure. Thacker *et al*. (2000a) showed that seroconversion can take at least three to six weeks to develop in experimentally infected pigs. Remarkably, no direct correlation has been found between the levels of serum IgG antibodies measured with the currently available serological tests and protection against *M. hyopneumoniae* challenge (Kobisch *et al*., 1993; Djordjevic *et al*., 1997).

### 1.2.3. Stress as an additional factor of immunomodulation

It has already been indicated that PEP can be exacerbated by several stressful events that the pigs might experience (Maes *et al*., 1996), such as crowding, mixing or sorting (Maes *et al*., 2008). In particular, the weaning process in commercial pig herds constitutes one of the most stressful events as piglets have to cope with: a) the abrupt separation from their sow, b) the handling by the farmers and the transportation, c) the establishment of a novel social hierarchy by being mixed with pigs of different litters, d) the provision of a different feed, and e) the exposure to different pathogens (Campbell *et al*., 2013; Bacou *et al*., 2017). However, in many herds, there are also other stressful events that occur during the suckling period, such as the castration of the pigs and adverse microclimate conditions in the production units (low or high ambient temperatures and humidity, aerial pollutants, etc.). Together with the weaning process, these events have the potential of creating a chronic stress situation for the pigs (Martínez-Miró e*t al*., 2016).

Additionally to the above, it is generally accepted that piglets reared under semi-feral extensive conditions are experiencing a lower degree of stress related to the weaning process, than the

piglets reared in commercial pig herds (Donaldson *et al*., 2002; Dudink *et al*., 2006). This is due to the fact that in semi-natural environments weaning is defined as a gradual process rather than a specific time, with the shift from the milk to other feed sources occuring between 12 and 17 weeks of age (Jensen and Recén, 1989; Stolba and Wood-Gush, 1989; Bøe, 1991). Additionally, in contrast with commercial pig herds, piglets have the opportunity to integrate with non-littermates prior to weaning.

It is well established that stress can suppress the immune system, leading to an increased incidence of infectious diseases (Thacker and Minion, 2012; Verbrugghe *et al*., 2012). Worsaae and Schmidt (1980) have found that piglets weaned at three weeks of age had higher plasma cortisol concentrations than pigs weaned at eight weeks of age. Cortisol is the primary glucocorticoid released during stress in swine and cattle (Griffin, 1989), hence it is one of the most commonly used stress biomarkers in these animals (Martínez-Miró e*t al*., 2016). Increased glucocorticoid concentrations during chronic stressful periods have been shown to increase the activity of immunosuppressive elements such as the regulatory T-cells (Kick *et al*., 2012). Additionally, *in vitro* experiments have shown that porcine lymphocyte cultures treated with cortisol exhibited reduced mitogen-stimulated blastogenesis (Kelley *et al*., 1982). This has also been observed *in vivo* where piglets weaned at two or three weeks of age exhibited lower mitogen-stimulated lymphocyte blastogenesis than those weaned at five weeks of age (Blecha *et al*., 1983). Collectively, these experiments showed that weaning together with chronic stressors that are commonly present in many herds can impair cell-mediated immunity, thus making pigs more susceptible to disease.

There are several studies that directly link the weaning process with the development of intestinal disease (mainly post-weaning *Escherichia coli* diarrhea and edema disease) during the nursery period (Pié *et al*., 2004; Moeser *et al*., 2007; Smith *et al*., 2010; Campbell *et al*., 2013).

Nevertheless, there are not a lot of published studies focusing on the impact of the weaning process on respiratory disease, and particularly PEP. de Groot *et al*. (2001) investigated the effect of mixing on specific long-term immune responses and protection against challenge infection after vaccination with pseudorabies virus (PRV). Mixed barrows showed suppressed cell-mediated and antibody-mediated immune responses after vaccination, as well as increased clinical signs after infection compared to the control barrows. Considering that *M. hyopneumoniae* is an important respiratory pathogen in PRDC (Brockmeier *et al*., 2002), these results highlight the need to elucidate whether the weaning process can influence *Mycoplasma*-induced disease and compromise the efficacy of vaccination schemes to control it.

## 1.3. EPIDEMIOLOGY OF *MYCOPLASMA HYOPNEUMONIAE* INFECTIONS

### 1.3.1. Occurrence

Infections with *M. hyopneumoniae* are present in almost all countries around the world where intensive pig production exists (Thacker and Minion, 2012). This has been further shown by either reporting the percentage of *M. hyopneumoniae* positive pigs by using several PCR detection methods (Table 1) or by reporting seroprevalence values. Indeed, *M. hyopneumoniae* has been detected by using nested PCR (nPCR) or real-time PCR on different samples obtained across different production periods in Canada, USA and several major pork producing EU countries, such as France, Germany, The Netherlands and Spain (Table 1).

Several studies have indicated high seroprevalence at slaughter age in several EU countries, with the average within-herd seroprevalence of *M. hyopneumoniae* being 62.9% in Germany, 70.8% in France, 79.0% in Belgium, 85.9% in Denmark and 94.7% in Italy (Andreasen *et al*., 2001; Meyns *et al*., 2011; Fablet *et al*., 2012; Merialdi *et al*., 2012; Nathues *et al*., 2014). Similarly, studies from Russia, China and Brazil have indicated seroprevalences of 31.7, 43.7 and 52.0%, respectively (He *et al.*, 2011; Kukushkin *et al.*, 2013; Vicente *et al.*, 2013).

Neverheless, there are some documented exceptions such as Finland and Switzerland, where regional and national eradication programs respectively have been applied, and resulted in PEP being only sporadically present (Rautiainen *et al*., 2001; Stärk *et al*., 2007). In Finland, within a two-year period, the eradication program applied resulted in an overall seroprevalence of 0.6% at slaughter and the lack of clinical cases of PEP in all participating herds (Rautiainen *et al*., 2001). In Switzerland, the number of clinical cases of PEP has dropped drastically from more than 200 in 2003 to eight in 2014, five in 2015 and three in 2016 (Overesch and Kuhnert, 2017).

**Table 1.** Detection rates of *M. hyopneumoniae* positive pigs by nPCR or real-time PCR across different age groups in several countries.

| Detection rates | nPCR (nasal swabs) | nPCR or real-time PCR (bronchial/tracheal swabs) | nPCR (BALF) | nPCR or real-time PCR (lung tissue) | Country | Study |
|---|---|---|---|---|---|---|
| **Suckling piglets** | | | | | | |
| 7.6-9.9% | + | | | | USA | Calsamiglia and Pijoan (2000) |
| 0.0-51.3% | + | | | | USA | Fano *et al* . (2007) |
| 0.0-6.4% | + | | | | Non-mentioned | Sibila *et al* . (2007a) |
| 12.3% | | | + | | Germany | Moorkamp *et al* . (2009) |
| 2.0% | | | | + | Germany | Nathues *et al* . (2010) |
| 1.7-22.1% | + | | | | 9 different EU countries | Villarreal *et al* . (2010) |
| **Nursery pigs** | | | | | | |
| 1.4-13.1% | + | | | | USA | Ruiz *et al* . (2003) |
| 3.8-4.4% | + | | | | Non-mentioned | Sibila *et al* . (2007b) |
| 3.3% | + | | | | Spain | Sibila *et al* . (2008) |
| 10.6% | | | + | | Germany | Moorkamp *et al* . (2009) |
| 9.3% | | | | + | Germany | Nathues *et al* . (2010) |
| 7.1-10.9% | | + | | | Belgium and Holland | Vangroenweghe *et al* . (2015) |
| **Fattening pigs** | | | | | | |
| 15.0-100.0% | | + | | | USA | Fano *et al* . (2007) |
| 29.8-45.5% | + | | | | Non-mentioned | Sibila *et al* . (2007b) |
| 60.0% | | + | | | France | Fablet *et al* . (2010) |
| 89.3% | | | | + | Canada | Charlebois *et al* . (2014) |
| 18.4-38.5% | + | | | | Belgium | Michiels *et al* . (2015) |
| 82.5% | | | + | | Belgium | Michiels *et al* . (2017) |
| **Sows** | | | | | | |
| 24.0-56.0% | + | | | | USA | Calsamiglia and Pijoan (2000) |
| 0.0-18.0% | + | | | | USA | Ruiz *et al.* (2003) |

There is no clear indication of age dependent susceptibility to *M. hyopneumoniae* infections (Maes *et al*., 2017). In this context, suckling, nursery and fattening pigs, as well as breeding animals (sows and boars) can be infected. Several field studies have indicated that the detection rates of *M. hyopneumoniae* positive pigs can vary between age groups within a herd (Table 1). Nevertheless, fattening pigs exhibit, in general, higher infection rates compared to suckling and nursery pigs (Table 1). These higher infection rates coincide with the fact that the clinical signs of PEP are mostly evident during the growing-finishing stages (Thacker and Minion, 2012).

It has been indicated that the prevalence of *M. hyopneumoniae* infections in suckling piglets determines to a large extent the clinical presentation and severity of PEP during the fattening period, especially in age segregated production systems (Fano *et al*., 2007; Sibila *et al*., 2007a). In fact, this is the reason why several eradication programs have focused on reducing *M. hyopneumoniae* infection levels prior to weaning, as usually infections further spread with aging of the pigs throughout the production process (Maes *et al.*, 1996; Frangman and Tubbs, 1997). It has also been observed that prevalence rates may vary between successive batches of suckling piglets within a herd (Table 1).

Studies have suggested that the prevalence of *M. hyopneumoniae* infections at weaning is a reflection of the number of sows that are shedding the pathogen, especially during the farrowing period (Calsamiglia and Pijoan, 2000; Ruiz *et al*., 2003). Although it is accepted that sows are commonly infected in areas of high pig density (Calsamiglia and Pijoan, 2000; Ruiz *et al*., 2003; Sibila *et al*., 2007a; 2007b; Grosse-Beilage *et al*., 2009), there are not a lot of studies shedding light on the dynamics of *M. hyopneumoniae* infections in the breeding sow population. Nevertheless, a longitudinal study by Pieters *et al*. (2014) showed that there can be within-herd variability in the percentage of infected sows between farrowing batches (0.0 to 48.0%) in herds weaning *M. hyopneumoniae* positive pigs.

### 1.3.2. Mode of transmission

Within herds, the transmission of *M. hyopneumoniae* mainly takes place through respiratory exudates and secretions that are spread through direct (nose-to-nose) contact between infected and susceptible animals (Thacker and Minion, 2012). Transmission through indirect contact by sharing the same airspace (aerosols spread between pigs of the same or different units within the herd) can also take place. However, the risk of having pigs infected after exposure to seropositive gilts was shown to be seven times higher by direct contact than by indirect contact (Morris *et al*., 1995).

In more detail, *M. hyopneumoniae* can spread within herds by two different ways:

1) **Vertical transmission**: piglets can become infected by their sow during the suckling period, by direct nose-to-nose contact (Sibila *et al*., 2009). Other ways of transmission between the sow and the piglet (i.e. in-utero or lactogenic) have not yet been documented. Young parity sows (parity 1-2) are more likely to transmit the pathogen to their offspring compared to older parity sows (parity >5; Fano *et al*., 2006). Nevertheless, there are exceptions where subpopulations of older parity sows can still serve as substantial shedders of *M. hyopneumoniae* (Calsamiglia and Pijoan, 2000; Grosse-Beilage *et al*., 2009).
2) **Horizontal transmission**: occurs when the pathogen spreads from infected to susceptible pigs, either directly by nose-to-nose contact or indirectly by sharing the same airspace (Maes *et al*., 2011). Direct nose-to-nose transmission can occur either between littermates suckling the same sow or between pigs of the same pen in the nursery and fattening units, and/or between pigs from adjacent pens within the same unit if no solid pen partitions are present. Research under experimental (Meyns *et al*., 2004) and field (Villarreal *et al*., 2011) conditions indicated that transmission of *M. hyopneumoniae* among pigs sharing the

same pens is slow, with adjusted reproduction ratios ($R_n$) showing that one infected pig can infect on average 0.56 to 1.16 susceptible penmates during a six-week nursery period.

Between herds, transmission of *M. hyopneumoniae* can occur by trade of (subclinically) infected animals or through airborne transmission (aerosols). Dee *et al*. (2009) and Otake *et al*. (2010) have reported aerosol transmission of *M. hyopneumoniae* to a distance of 4.7 and 9.2 km, respectively, from the source populations. In the latter case, recovered air samples were shown to be infectious when experimentally inoculated to naïve pigs. Airborne transmission poses a risk for *M. hyopneumoniae*-free SPF herds to become re-infected, especially in areas with high pig (herd) density (Thomsen *et al*., 1992).

Wild boars have been shown to be infected with *M. hyopneumoniae*, but these infections are mostly subclinical (Sibila *et al*., 2010) and there is no clear indication that these animals can serve as a reservoir of infections for commercial swine herds. In contrast, it has been proposed that wild boars in the vicinity of the swine herds are the recipients rather than the transmitters (Kuhnert and Overesch, 2014).

Little research has been performed about the role of fomites and personnel as mechanical vectors for the transmission of the pathogen. Nevertheless, when standard hygiene and biosecurity protocols are applied, the spread of *M. hyopneumoniae* by fomites and personnel to naïve pig populations can be prevented (Batista *et al*., 2004; Pitkin *et al*., 2011).

Once infected, pigs can carry the pathogen in their lungs and transmit it to naïve pigs for a long period of time, even without the presence of apparent clinical signs (between 214 and 240 days after infection; Pieters *et al*., 2009; 2010).

### 1.3.3. Factors influencing the dynamics of infection

There are several factors that majorly influence the dynamics of *M. hyopneumoniae* infections in pig herds. Table 2 presents a list of these risk factors. The levels of infection during the peri-weaning period have gained significant attention during the last years (Table 1). Transmission during this period is influenced by several overlapping factors, namely the type of production system (Maes *et al*., 2017), housing and management conditions inherent to each herd (Table 2) and also, the infection status of the breeding sow population (Stärk et al., 2000; Garza-Moreno *et al*., 2018). In age segregated systems with multi-site production, the sows are considered to be the reservoir of *M. hyopneumoniae* infections for the suckling and recently weaned piglets (Nathues *et al*., 2013a). In continuous flow systems, horizontal transmission through contact with different age groups is probably also a major determinant in *M. hyopneumoniae* transmission (Giacomini *et al*., 2016). Once infected, peri-weaned piglets can spread the pathogen to other pigs within their age group during the nursery and fattening periods.

Finally, the dynamics and/or clinical outcome of *M. hyopneumoniae* infections are also influenced by factors related to the pathogen itself. A recent study by Michiels *et al*. (2017) found that the prevalence and severity of *Mycoplasma*-like lung lesions at slaughter were significantly higher in batches of slaughter pigs where more different *M. hyopneumoniae* strains were found. Additionally, experimental studies have shown that after inoculation highly virulent strains were able to transmit faster and also, induce lung lesions and clinical disease earlier than the low virulent strains (Meyns *et al*., 2004; 2007; Villarreal *et al*., 2009). Another factor contributing to *Mycoplasma*-induced disease is the infectious dose. Although it is not easy to evaluate under field conditions, several experimental studies have shown that high infectious doses can induce clinical disease relatively quickly, while low doses induce no or only minimal clinical signs (Stevenson 1998, Fano *et al*., 2005a).

**Table 2.** Risk factors for the occurrence and transmission of *M. hyopneumoniae* infections.

| Herd characteristics | Comments | Studies |
|---|---|---|
| Size of the herd | Large mixed breeding-finishing herds exhibit a higher risk to become (re)-infected | Hege *et al* ., 2002 |
| Number of crates per farrowing unit | High number of farrowing crates within single units (> 16) increase the risk of piglets being already infected at weaning | Nathues *et al* ., 2013a |
| Fattening herds | Fattening herds that buy pigs from multiple sources are more likely to be (re)-infected | Hege *et al* ., 2002; Maes *et al* ., 2008 |
| Regional pig density / neighbouring pigs herds | Regions with high pig density and presence of infected neighbouring herds increase the risk of transmission between herds | Goodwin, 1985; Hege *et al* ., 2002; Dee *et al* ., 2009 |
| **Management practices** | **Comments** | **Studies** |
| All-in/all-out *versus* continuous production flow | All-in/all-out across all production stages is linked with lower infection pressure compared to continuous flow. Additionally, with delayed transmission across age groups | Flesjå and Solberg, 1981; Clark *et al* ., 1991; Morris *et al* ., 1995; Nathues *et al* ., 2013a |
| Separation of production sites | Multi-site operations compared to single-site farrow-to-finish herds show a lower infectious pressure during the nursery period | Sibila *et al* ., 2009; Maes *et al* ., 2011 |
| Purchase policy | High sow replacement rates, purchasing gilts from multiple sites and/or purchasing gilts of unknown infectious status are major risk factors for the introduction and/or maintainance of the infections | Maes *et al* ., 2000; Nathues *et al* ., 2013a |
| Gilt acclimatization | When gilts are not exposed to the farm-specific germ flora through contact with living pigs during the quarantine period, the chance of detecting positive piglets shortly after weaning nearly doubles | Moorkamp *et al* ., 2009; Nathues *et al* ., 2014 |
| Parity segragation | Separating gilts and their piglets from older parity sows until reaching their second gestation lowers the infectious pressure in the sow population | Maes *et al* ., 2017 |

| Management practices | Comments | Studies |
|---|---|---|
| Duration of lactation period | Longer lactation periods are linked to higher risk of vertical transmission and additionally, higher infectious pressure in the fattening period. | Maes *et al* ., 2008; Nathues *et al* ., 2014 |
| Cross-fostering | Cross-fostering increases the risk of piglets being infected at weaning | Nathues *et al* ., 2013b; Maes *et al* ., 2017 |
| Hygiene and internal biosecurity | Cleaning and disinfecting between batches, sufficient empty period in the farrowing units, isolating sick pigs in hospital pens and promptly euthanizing terminally sick pigs reduce: a) the risk of piglets being infected at weaning and, b) the risk of herds to be (re)-infected | Pitkin *et al* ., 2011; Nathues *et al* ., 2013a, Simionatto et al., 2013 |
| Stocking densities | High densities are linked with a higher infectious pressure compared to low densities | Clark *et al* ., 1991; Maes *et al* ., 1996; 2008 |
| Air-filtration systems | Installation of air filtration systems prevents units being close to infected source populations from becoming (re)-infected | Dee *et al* ., 2010 |
| Mycotoxins | Feeding fumonisin B-contaminated feed is linked to more severe clinical disease and lung lesions. This was not observed with deoxynivalenol-contaminated feed | Pósa *et al* ., 2013; Michiels *et al* ., 2018 |
| **Environmental conditions** | **Comments** | **Studies** |
| Air volume and indoor ventilation rates | Low airspace (< 3 $m^3$ air volume per pig) and/or low ventilation rates (< 60 $m^3$ per hour per pig) increase the severity of clinical disease and lung lesions | Flesjå *et al* ., 1982; Stärk *et al* ., 2000 |
| Microclimate of the buildings | Non-optimal indoor temperaratues (including fluctuations) and high air humidity increase infectious pressure and the severity of disease | Done *et al* ., 1991; Stärk *et al* ., 2000; Nathues *et al* ., 2013b |
| Outdoor weather conditions | Winter weather and high outdoor relative humidity increase infectious pressure and the severity of clinical disease | Goodwin, 1985; Maes *et al* ., 2000; Vangroernweghe *et al* ., 2015 |
| Ammonia and dust | High indoor ammonia and dust concentrations are linked to increase severity of clinical disease | Maes *et al* ., 1996; 2017 |

### 1.3.4. Role of maternal immunity

The role of maternal immunity in the protection of piglets has not yet been fully elucidated and remains a controversial matter. The median half-life of maternally derived antibodies against *M. hyopneumoniae* in the serum of piglets has been suggested to be 15.8 days (Morris *et al*., 1994). The persistence of these antibodies is shown to vary among litters and depends on the levels of antibodies in the colostrum of the sows and colostrum intake by the piglets (Wallgren *et al*., 1998; Rautiainen and Wallgren, 2001). It has been indicated that maternally derived antibodies only provided partial protection against lung lesion development after experimental challenge infection and that it had a limited-to-no effect on preventing the animals from becoming infected (Thacker *et al*., 2000b). Similarly, under experimental conditions, Djordjevic *et al*. (1997) showed that IgG antibody concentrations (in serum and respiratory tract washings of finishing pigs) were not correlated with protection against challenge infection.

Bandrick *et al*. (2008) showed that lymphocytes that were passively transferred from vaccinated sows to four-day-old piglets *via* the colostrum, were able to proliferate and participate in a functional response against *M. hyopneumoniae*. Assessments were done *in vivo* and *in vitro via* delayed-type hypersensitivity testing and lymphocyte stimulation, respectively. Nevertheless, the significance of these findings on protection against challenge infection was not investigated.

## 1.4. CLINICAL DISEASE AND PORCINE RESPIRATORY DISEASE COMPLEX

### 1.4.1. Clinical signs and performance losses

In herds endemically infected with *M. hyopneumoniae*, PEP is characterized by low mortality and high morbidity (Sarradell *et al*., 2003). The main clinical signs of PEP in these herds are dry and intermittent coughing, together with reduced growth rates. Dry and non-productive coughing appears slowly and mostly affects pigs older than six weeks of age. Nevertheless, clinical signs can also appear in animals as young as three or four weeks of age (Maes *et al*., 1996; Sibila *et al*., 2009). Reports have indicated that the peak of coughing usually appears three to five weeks after initial infection (Ciprián *et al*., 1988, Vicca *et al*., 2003), but under field conditions this depends also on the percentage of pigs infected with *M. hyopneumoniae* in the affected age groups, the presence of other respiratory pathogens, and also the management and environmental conditions in the herd (Table 2; Pijoan, 2005; Sibila *et al*., 2009; Van Alstine, 2012). Coughing tends to decrease progressively after eight weeks post-infection or can persist longer, depending on the aforementioned factors.

There are also cases where endemically infected herds present a subclinical form of the disease. In these cases, animals are infected with *M. hyopneumoniae*, but do not show any coughing or only show intermittent and mild coughing for several weeks (Regula *et al*., 2000; Desrosiers, 2001; Maes *et al*., 2017). Nevertheless, this form of PEP is still inducing financial losses to the herds as well as compromises animal welfare, since it causes growth retardation and predisposes the animals to secondary bacterial pathogens.

At this point, it should be mentioned that in the vast majority of the affected herds, PEP is a heterogeneous disease that results from the combination of *M. hyopneumoniae* as primary etiological agent and other bacterial pathogens that are secondarily involved (Thacker and Minion, 2012), such as *Bordetella bronchiseptica* (*B. bronchiseptica*), *Haemophilus parasuis* (*H.*

*parasuis*), *Pasteurela multocida* (*P. multocida*), *Streptococcus suis* (*S. suis*), *Trueperella pyogenes* (*T. pyogenes*) and *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*). *M. hyopneumoniae* can potentially trigger these pathogens to act synergistically and cause more severe clinical signs, such as productive cough, high fever, reduced appetite, labored breathing, or thumping and prostration (Maes *et al*., 1996).

Several studies have provided evidence of a reduction in the ADG, especially from the early fattening period onwards (Straw *et al*., 1989; Rautiainen *et al*., 2000, Regula *et al*., 2000). Additionally, growth retardation is usually linked with a decreased feed conversion efficiency and unevenness in weights between pigs of the same age groups. According to the literature review of Straw et *al*. (1989) that utilized data from 24 different studies, ADG decreases by 37.4 g for every 10% of the lung surface presenting *Mycoplasma*-like lung lesions. Moreover, feed conversion efficiency decreased on average by 14%. Sitjar *et al*. (1996) showed that the growth of pigs infected with *M. hyopneumoniae* later during the fattening period was less compromised than that of pigs infected earlier in the fattening period.

In herds that are immunologically naïve, the results of the introduction of *M. hyopneumoniae* can be devastating (Thacker and Minion, 2012). In these herds, pigs of all age groups are susceptible and the disease spreads relatively rapidly. Morbidity rates can reach 100% and the clinical signs include severe coughing, labored breathing, fever and increased mortality rates.

### 1.4.2. Macroscopic lung lesions

The macroscopic lung lesions following infections with *M. hyopneumoniae* consist of red to purplish consolidated areas that are mainly located in the apical and cardiac lobes, and less commonly at the cranial parts of diaphragmatic lobes. These areas are often well demarcated and are raised above the surface or at the surface of the rest of the lung (Figure 3; Kobisch and Friis, 1996; Garcia-Morante *et al*., 2016). Upon incision, these areas have a meaty consistency and often

there is presence of catarrhal exudate (mucous) in the airways. Overall, these lesions are called 'active lesions' and are observed during the early and middle stages of infection.

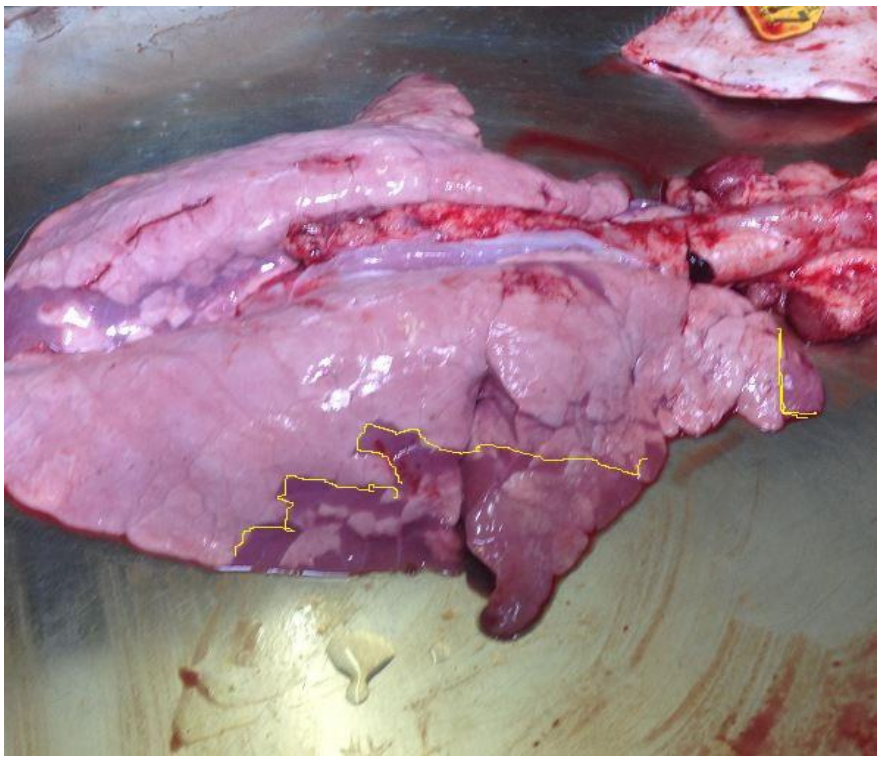


**Figure 3**. Lungs of a pig experimentally infected with *M. hyopneumoniae*. The yellow lines show the well demarcated lesions of red to purple consolidation in the apical, the cardial and the diaphragmatic lobes of the right lung.

Experimental studies have shown that 'active lesions' may appear at around seven days after challenge infection and reach their maximal size at about four weeks after infection (Kobisch *et al*., 1993; Maes *et al*., 1996). After a period of five to six weeks post-infection, these lesions start regressing and by seven to 10 weeks post-infection the lungs already show 'recovery lesions' (Kobisch *et al*., 1993). These lesions are called fissures, and they are formed during the chronic stages of infection. Fissures are purplish to grey interlobular connective tissue formations (scars), that are shrunk below the surface of the lobes (Kobisch and Friis, 1996). Additionally, they have a

more solid texture than the unaffected neighboring parenchyma. Under experimental conditions, fissures almost completely disappear by 12 weeks post-infection (Strasser *et al*., 1992; Sørensen *et al*., 1997).

As mentioned above (subsection **1.4.1. Clinical signs**), under field conditions PEP is the result of mixed infections with *M. hyopneumoniae* and other pathogens that are secondarily involved. In these situations, and especially if *P. multocida*, *S. suis* and *T. pyogenes* are involved (alone or in combination), the lung lesions can have a more firm consistency and a more greyish color (Thacker and Minion, 2012; Maes *et al*., 2017). Additionally, the lesions are diffused in a larger area of the lung and mucopurulent exudate is often present in the airways.

Last but not least, although cranioventral lung consolidation is well known to be linked to *M. hyopneumoniae* infections and PEP, SIV has also been shown to cause similar lung lesions (Khatri *et al*., 2010). Thus, macroscopic lung lesions are not considered to be a pathognomonic diagnostic criterion for *M. hyopneumoniae* infections.

### 1.4.3. Porcine respiratory disease complex

Under modern farming conditions, pneumonia is most commonly the result of interactions between multiple respiratory pathogens (Van Alstine, 2012). The intensification of commercial pork production has facilitated respiratory disease transmission by increasing the pig population size and density (Jones *et al*., 2013). Many non-infectious factors that predispose to respiratory disease became more difficult to control the last few decades (Brockmeier *et al*., 2002). These are the management of high stocking densities in the breeding units as well as the nursery and fattening units, the maintenance of proper room temperatures and ventilation rates and also, the removal of aerial pollutants (e.g. ammonia, dust and micro-organisms). Moreover, biosecurity management has also become more complex, given that large numbers of pigs (sometimes a thousand or more) originating from different breeding units are often mixed together, and that

large numbers of vehicles and personnel visit the herds (sometimes even on a daily basis; Jones *et al*., 2013). Consequently, a drastic increase in the prevalence of respiratory diseases has occurred and in many herds it is not uncommon to find multiple respiratory pathogens circulating at any given time. The term porcine respiratory disease complex (PRDC) has been used to describe pneumonia of polymicrobial and multifactorial etiology (Brockmeier *et al*., 2002; Thacker, 2002).

A large number of respiratory pathogens are involved in the etiology of PRDC. These pathogens can be divided into primary and secondary (Bochev, 2007). Briefly, primary pathogens are able to evade and disrupt the immune responses of the host, and establish disease on their own. Secondary pathogens are usually opportunistic pathogens that require the presence of predisposing factors in order to cause respiratory disease (Van Alstine, 2012). These factors are not only improper management and environmental conditions, but also the presence of primary pathogens. Figure 4 shows the different primary and secondary pathogens participating in PRDC.

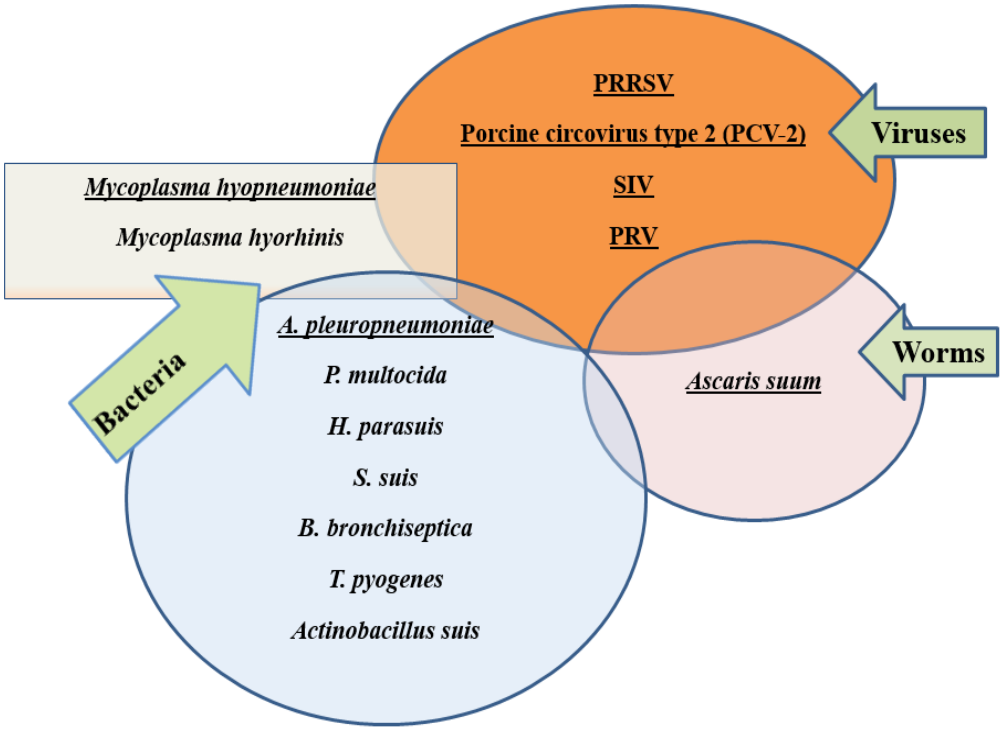



**Figure 4**. Primary and secondary pathogens involved in PRDC. The primary pathogens are underlined in order to be distinguished from the secondary ones.

There are several experimental and field studies that have demonstrated the interactions of *M. hyopneumoniae* with other pathogens involved in the PRDC (Table 3). These interactions show that *M. hyopneumoniae* apart from being the primary etiological agent of PEP, has a more broad role in the development of respiratory disease. This is by having either a potentiating or an additive effect to other primary or secondary respiratory pathogens. All these studies have also highlighted that there are still different combinations between *M. hyopneumoniae* and other pathogens that are involved in PRDC, which have not yet been elucidated.

**Table 3**. Experimental or field studies showing a potentiating or additive effect of *M. hyopneumoniae* to other respiratory pathogens.

| Interactions investigated | Interactions | Study |
|---|---|---|
| *M. hyopneumoniae* and PCV-2 | *M. hyopneumoniae* increased the overall severity of macroscopic lung lesions in a dual-infection model as well as the PCV2-associated microscopic lesions in the lung and lymphoid tissues. It also increased the incidence of post-weaning multisystemic wasting syndrome. | Opriessning *et al* ., 2004 |
| *M. hyopneumoniae* and PCV-2 | PCV-2 infected pigs were more likely to be infected with *M. hyopneumoniae* compared to PCV-2 negative pigs. | Dorr *et al* ., 2007 |
| *M. hyopneumoniae* and *A. pleuropneumoniae* | *M. hyopneumoniae* increased the severity of lung lesions and clinical disease associated with *A. pleuropneumoniae* serotype 9. | Marois *et al* ., 2009 |
| *M. hyopneumoniae* and PRRSV | *M. hyopneumoniae* increased the severity and duration of PRRSV-induced pneumonia independent of the timing of infection with either pathogen. | Thacker *et al* ., 1999 |
| *M. hyopneumoniae* and PRV | PRV increased the extent and prevalence of lung lesions induced by *M. hyopneumoniae*. | Shibata *et al* ., 1998 |
| *M. hyopneumoniae* and SIV | *M. hyopneumoniae* increased the severity of clinical disease and lung lesions induced by the H1N1 subtype of SIV. | Deblanc *et al* ., 2012 |

## 1.5. DIAGNOSIS

The anamnesis and the clinical signs present are useful for making a tentative diagnosis, but are not pathognomonic for *M. hyopneumoniae* infections (Maes *et al*., 1996; Maes *et al*., 2017). The same applies for the microscopic and macroscopic lung lesions, as these lesions can be similar to lesions induced by other pathogens. Thus, in practice an accurate and conclusive diagnosis is established by the use of several laboratory techniques, which can demonstrate whether *M. hyopneumoniae* is (one of) the causative agent(s) of the observed clinical signs and lung lesions.

In endemically infected herds, the accurate diagnosis of *M. hyopneumoniae* infections requires the combination of different methods. These are the clinical examination (assessing cough severity and growth retardation) and the macroscopic evaluation of the lung lesions at slaughter, together with the use of laboratory techniques, such as serology and PCR detection methods. The reason is that diagnostics are not only used to establish the presence of the pathogen, but also to uncover the dynamics of infection across the different age groups and production sites (Desrosiers, 2001; Thacker, 2004). In other words, it is important to identify at which production phase the animals are becoming infected and whether the herd is clinically or subclinically infected. Additionally, this approach allows to identify on which production phase the *M. hyopneumoniae* infection has its largest impact. In that way, the herd veterinarian can decide the most appropriate prevention and control strategies. For example, the herd veterinarian can choose whether the animals need to be treated with antimicrobials or not, at which production phase the animals should be vaccinated and the method of choice for acclimatizing the replacement gilts. Also, in case eradication is an option, this information can help to determine the most appropriate eradication protocol.

In this context, there have been some efforts to utilize diagnostics in order to create a system which classifies the herds according to their infection status (Garza-Moreno *et al*., 2018). This system can be applied in gilt rearing sites, breeding herds, farrow-to-finish herds or only-fattening

herds, whether or not these production facilities are part of single-site or multi-site production operations. According to a combination of different diagnostic methods, it classifies the herds in four different categories: a) negative herd, b) provisionally negative herd, c) positive herd – subclinically infected (subdivided in categories I and II), and d) positive herd – clinically affected (Table 4).

**Table 4**. Classification of farms according to their *M. hyopneumoniae* infection status (table obtained from Garza-Moreno *et al*., 2018).

| Classification | | Monitoring *M. hyopneumoniae* status | | | |
|---|---|---|---|---|---|
| | | Observational diagnosis | | Laboratory diagnosis | |
| | | Clinical signs | Lung lesions | ELISA* | PCR result |
| Negative | | Not observed | Not observed | Negative | Negative |
| Provisionally negative | | Not observed | Not observed | Positive | Negative |
| Positive | Subclinically infected I | Not observed | Not observed | Positive / Negative | Positive |
| | Subclinically infected II | Not observed | Observed | Positive / Negative | Positive |
| | Clinical affected | Observed | Observed | Positive / Negative | Positive |

*ELISA results will be dependent on infection dynamics, sampling time point and if the vaccination against *M. hyopneumoniae* is applied.

It should be mentioned though, that under field conditions there are numerous factors that can complicate the application of such classification systems and/or the attempts to monitor the infection status of the herds. One of these factors, is the type of sampling. Different sampling methods can be applied, such as single-point sampling (same time point – same age group), cross-sectional sampling (same time point – different age groups) or longitudinal sampling (different time points – same or different age groups). The type of sampling affects the accurate evaluation of the prevalence of disease at herd level. Single-point sampling is very cost effective, but it does not take into account the prevalence of *M. hyopneumoniae* infections in other age groups. Cross-

sectional sampling allows to assess the prevalence of *M. hyopneumoniae* infections in different age groups, but does not provide a long-term picture of the infection dynamics in the herd. Longitudinal sampling is more time consuming and more laborious than the previous sampling methods, but can provide more information on when the animals are becoming infected and how the percentage of *M. hyopneumoniae* positive pigs deviates between batches.

A second factor that could affect the ability to monitor the *M. hyopneumoniae* infection status of a herd is the sample size (Calsamiglia *et al*., 1999; Fosgate, 2009). Proper sample sizes provide the ability to have a more accurate image of the prevalence of *M. hyopneumoniae* infections. For that reason, Galina and Clavijo (2016) have indicated that for classifying the peri-weaned piglets of a breeding herd as positive – subclinically infected (II), less than 10% of peri-weaned pigs should be *M. hyopneumoniae* positive by nPCR in four consecutive samplings, of 45 laryngeal swabs each (i.e. piglets of four different batches are sampled every 30 days at weaning). Other factors that could complicate the attempts to monitor the infection status of the herds are the sensitivity and specificity of the diagnostic methods applied, the sampling site, and also the time point at which the animals are becoming infected (Gardner and Blanchard, 2006). More details about these factors are provided in the following paragraphs.

### 1.5.1. Clinical signs

To evaluate coughing severity among the different age groups present in the herd, pigs need to be observed over a considerable time-period and should be encouraged to move. Nevertheless, it should be mentioned that coughing has a very low diagnostic sensitivity. Sørensen *et al*. (1993) indicated that using cough evaluation as the sole diagnostic method failed to detect 30% of *M. hyopneumoniae* infected herds. Thus, the evaluation of coughing severity needs to be combined with laboratory techniques.

Two different scoring systems for evaluating coughing have been described by Maes *et al*. (1999) and Nathues *et al*. (2012). The first one uses the following formula to calculate the percentage of pigs in a pen that cough over a period of 10 minutes: **[(number of pigs coughing during 10 minutes / total number of pigs in that pen) x 100]**. The latter one uses the following formula to calculate the average percentage of pigs in a pen coughing per minute of observation: **[total number of coughing bouts / (total number of pigs in that pen x total time of observation)]**. The coughing scores obtained by either system can be further used to extract an average score that represents all pens of a nursery or a fattening unit. In that way, herd veterinarians can monitor how coughing evolves between the different production batches, and assess the efficacy of the different prevention and control strategies. Currently, there is also the development of electronic systems that generate coughing scores by the use of microphones, nevertheless at this stage their market penetration remains limited (Berckmans *et al*., 2015).

Nathues et al. (2012) indicated that a quantitative evaluation of the onset of non-productive cough in fattening pigs can improve the diagnosis of PEP when combined with serology, and can occasionally substitute the detection of *M. hyopneumoniae* by PCR. Nevertheless, these results should be interpreted with caution. As mentioned previously, in many herds there is co-circulation of other respiratory pathogens that can cause productive cough instead of the typical dry and non-productive cough that typically exists in uncomplicated *M. hyopneumoniae* infections.

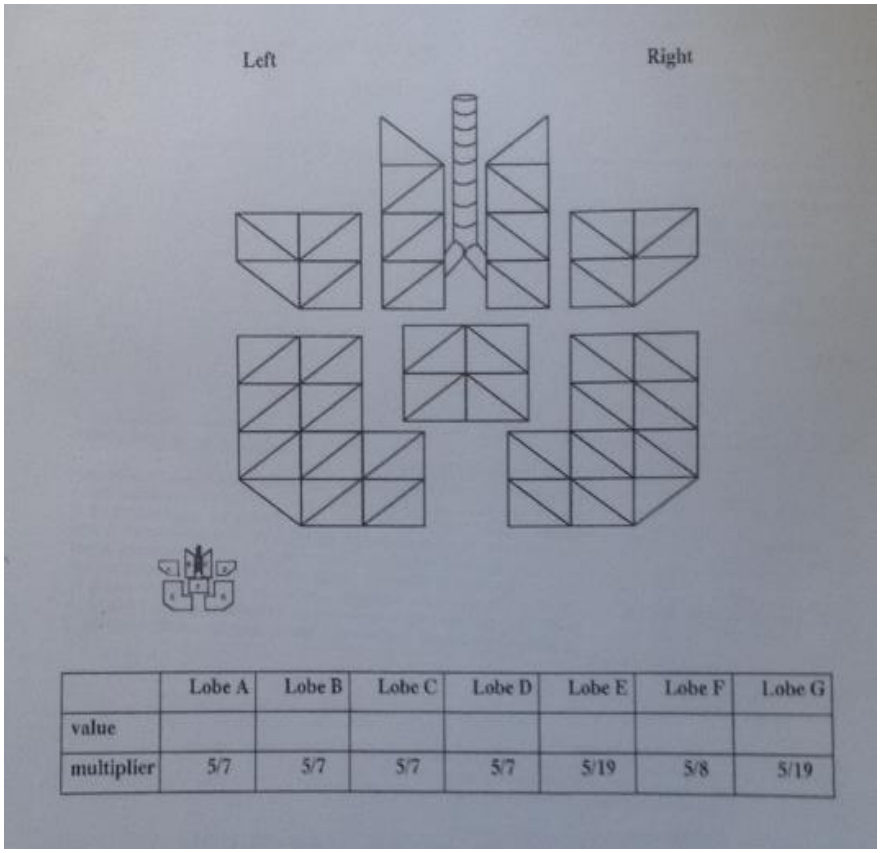
### 1.5.2. Macroscopic lung lesions

The macroscopic evaluation of the lung lesions at slaughter is commonly used to quantify the presence of lung consolidation at batch level, which is suggestive of PEP (Thacker, 2004). Several different scoring systems have been described, based on the visual examination and palpation of the lungs at the slaughter line (Madec and Kobisch, 1982; Morrison *et al.*, 1985; Straw *et al.*, 1986; Christensen *et al.*, 1999). Briefly, these systems are used to estimate the average total

surface area or the average total weight percentage of the lung parenchyma affected in the batches scored (by entering the individual lung scores and averaging them), as well as the prevalence of lungs showing *Mycoplasma*-like lung lesions.

However, the quantification of lung consolidation at slaughter poses several limitations for the adequate diagnosis of *M. hyopneumoniae* infections. Firstly, as mentioned previously, there can be several other pathogens that can cause similar lesions, such as SIV or combined infections with PRV and *P. multocida* (Fuentes and Pijoan, 1987; Khatri *et al*., 2010). Secondly, *M. hyopneumoniae*-induced 'active lung lesions' might have healed by the time of slaughter, hence leading to false negative diagnoses (Kobisch *et al*., 1993). Thirdly, the presence of pleurisy induced by pathogens such as *A. pleuropneumoniae* and *H. parasuis* can 'mask' the presence of consolidated areas in the lung (Sibila *et al*., 2009). Fourthly, there can be practical limitations that have to do with the slaughterhouse, such as the possibility of the batch that needs to be scored to be mixed with other batches or the fast speed of the slaughter line which sometimes does not allow to properly evaluate each lung. Last but not least, the experience of the examiner also plays an important role. Garcia-Morante *et al*. (2016) reported that evaluating the extent of lung consolidation may vary between different examiners.

In the present thesis, two different scoring systems were used: a) the system of Hannan *et al*. (1982) which is suitable for experimental studies, and b) the system of Morrison *et al*. (1985), as modified by Del Pozo Sacristán *et al*. (2012a; 2014), which is suitable for slaughterhouse investigations. Briefly, in the system of Hannan et *al*. (1982) every affected area of the lung is drawn in a diagram that divides the surface area of lung into 57 triangles (Figure 5). Then, the number of triangles affected in each lobe are multiplied by five and divided by the number of triangles representing the lobe. Then, each lobe score is added to provide an overall area lung score that quantifies the total area of the lung parenchyma affected.




| | Lobe A | Lobe B | Lobe C | Lobe D | Lobe E | Lobe F | Lobe G |
|---|---|---|---|---|---|---|---|
| value | | | | | | | |
| multiplier | 5/7 | 5/7 | 5/7 | 5/7 | 5/19 | 5/8 | 5/19 |

**Figure 5**. Form used in the scoring system of Hannan *et al*. (1982). The total surface area of the lung is divided into 57 triangles.

The modified score of Morrison *et al*. (1985) used in this thesis estimates the total weight percentage of the lung parenchyma affected, with the different lobes representing the following weight percentage: apical (10%), cardiac (7%), accessorial (6%) and diaphragmatic (30%). The percentage of affected surface area of each lobe is multiplied by the lobe's relative weight. Then, each lobe score is added to provide the total weight percentage of affected lung.

### 1.5.3. Microscopic lung lesions

The basic histological examination of formalin-fixed, paraffin-embedded lung tissue sections is not commonly used to diagnose *M. hyopneumoniae* infections under field conditions. The main reason is its low sensitivity as a diagnostic method. Armstrong *et al*. (1984) used 100 lungs from

five different herds and found that about one-third of the lungs having microscopic lung lesions typical of *M. hyopneumoniae* were negative by indirect IF staining and culture of tissue samples. Histological evaluation of lung lesions can however be very valuable in experimental studies, where there is controlled exposure to *M. hyopneumoniae* (Vicca *et al*., 2003; Meyns *et al*., 2004; 2007; Del Pozo Sacristán *et al*., 2012b). In these studies, it is used to quantify and compare the severity of peribronchiolar and perivascular lymphohistiocytic infiltration, and nodule formation, between the different treatment groups employed.

### 1.5.4. Laboratory techniques showing the presence of *M. hyopneumoniae*

***Culture and isolation of the pathogen***

The 'gold standard' diagnostic technique for the detection of a bacterial pathogen is its isolation from fresh lung tissue or BALF by bacteriological culture (Thacker and Minion, 2012). Nevertheless, as mentioned before (section **1.1. Characteristics of *M. hyopneumoniae***), culturing of *M. hyopneumoniae* is a laborious and extremely slow process, that requires the use of special media. Additionally, cultures can be frequently overgrown by *M. hyorhinis, M. flocculare* or other microbial organisms which are commensal pathogens of the respiratory tract. Hence, this laboratory technique is considered to be impractical under field conditions.

***Fluorescent antibody and immunohistochemistry assays***

Immunofluorescence (IF) or immunohistochemistry (IHC) have been used to detect the presence of *M. hyopneumoniae* antigen in lung tissue sections (Thacker, 2004). These techniques are not commonly used anymore under field conditions, since they have several disadvantages. Firstly, the diagnosis can only be made postmortem and not in live animals. Secondly, the tissue sections used need to include airways with ciliated epithelia. Thus, only small portions of the lung sample are tested, increasing the likelihood to obtain a false negative result. Thirdly, the collected lung tissue needs to be snap frozen with polythelene glycol (IF) or fixed in 10% formalin solution

(IHC) soon after death, in order to avoid the degradation of the ciliated epithelia (Thacker and Minion, 2012). Particularly for the IF, it has been indicated that a positive result is associated with the early stages of pneumonia, when high numbers of *M. hyopneumoniae* are present in the lungs. In contrast, low sensitivities are observed during the chronic stages of pneumonia. Sørensen *et al*. (1997) reported a sensitivity of 96% up to 28 days post-infection, which then dropped to 73 and 18% at 57 and 85 days post-infection, respectively.

In this thesis, an indirect IF assay was used in the experimental study to semi-quantitatively assess the number of *M. hyopneumoniae* organisms in the airways. Briefly, this was done by using the scoring system of Kobisch *et al*. (1978), which assigns four different scores depending on the intensity of the IF staining (i.e. 0 which corresponds to no IF; 1 corresponds to limited IF; 2 corresponds to moderate IF; 3 corresponds to intense IF). This scoring system has also been used in other experimental studies (Vicca *et al*., 2003; Meyns *et al*., 2004; 2007; Del Pozo Sacristán *et al*., 2012b).

***Serology***

Nowadays, the enzyme-linked immunosorbent assay (ELISA) is the most commonly used serological assay to measure the levels of circulating IgG antibodies against *M. hyopneumoniae* (Ameri *et al*., 2006). Commercial blocking or indirect assays are commonly used in both field and experimental conditions (Thacker, 2004; Gomes Neto *et al*., 2014). The main advantages of these assays are that a high number of samples can be analyzed quickly and with a low cost, and that quantitative results can be obtained.

However, the interpretation of these results can be challenging. The currently available serological assays cannot distinguish maternally derived antibodies or antibodies generated from infection or vaccination (Maes *et al*., 2017). Hence, under field conditions veterinarians often need to either perform a cross-sectional or a longitudinal sampling in order to obtain more information about the

origin of the antibodies. Moreover, the time by which infected animals seroconvert can be very variable. Experimental studies have shown that it can take between three to eight weeks for the majority of the animals in a challenge infected group to seroconvert (Kobisch *et al*., 1993; Fano *et al*., 2005b). Additionally to the above, several studies have shown that the sensitivity of the different commercial ELISA assays that are widely used in field and experimental conditions can be very low, ranging between 37 and 49% (Erlandson *et al*., 2005). Due to the aforementioned reasons, ELISA assays should be used to monitor *M. hyopneumoniae* infections at batch or herd level, and not in individual animals. Finally, it has been shown that there can be extensive cross-reactivity between *M. hyopneumoniae* and other mycoplasmas, such as *M. flocculare* and *M. hyorhinis*, which can produce low positive signals (Ameri *et al*., 2006; Gomes Neto *et al*., 2014).

***PCR detection methods***

Nowadays, nPCR or real-time PCR are two of the most commonly used laboratory techniques, that enable the accurate detection of the pathogen (Thacker, 2004). These techniques possess a lot of advantages, such as the ability to analyze a high number of samples in a short period of time and with a reasonable cost. Additionally, a wide variety of sample types can be used, and both live and dead animals can be sampled (Sibila *et al*., 2009). Moreover, both techniques offer a high sensitivity. It has been indicated that nPCR can detect as low as 1 *fg* of *M. hyopneumoniae* chromosomal DNA (equivalent to approximately one genome) per PCR reaction (Kurth *et al*., 2002). Concerning the real-time PCR, the limit of detection can be as low as 1 to 6.5 *fg* of *M. hyopneumoniae* chromosomal DNA per PCR reaction (Dubosson *et al*., 2004; Marois *et al*., 2010). A further advantage of real-time PCR over nPCR is that it offers the possibility to quantify the amount of DNA in the samples and report it either as genome copies/mL (Pulgarón *et al*., 2015) or convert it to Log copies of *M. hyopneumoniae* organisms/mL (Marois *et al*., 2010).

Nevertheless, these techniques also possess some disadvantages, such as the fact that their high sensitivity in detecting the pathogen can lead to false positive results when pipetting procedures are not optimal (Thacker and Minion, 2012). Also, they can amplify DNA from both live and dead *M. hyopneumoniae* cells, which can raise the question of whether they detect bacterial detritus, rather than active *M. hypneumoniae* infection (Sibila *et al*., 2009). Finally, some of these techniques can provide false negative results (Dubosson *et al*., 2004; Strait *et al*., 2008), due to the use of only one DNA target sequence for amplification and the presence of genomic differences among the different strains of *M. hyopneumoniae*.

Both nPCR or real-time PCR can be performed on a wide variety of samples, such as lung tissue, BALF, tracheobronchial washings, tracheobronchial swabs, laryngeal swabs, oropharyngeal brushing samples, nasal swabs and oral fluids (Marois *et al*., 2007; Fablet *et al*., 2010; Del Pozo Sacristán *et al*. 2012a; 2014). Regarding the sensitivity of the different sample types, it is generally accepted that lower respiratory tract samples (i.e. lung tissue including bronchioles or sampling at tracheobronchial sites) offer better detection rates than upper respiratory tract samples (i.e. laryngeal swabs, oropharyngeal brushing samples, nasal swabs). However, a recent study by Pieters *et al*. (2017) indicated that during the early stages of infection laryngeal swabs offered better detection rates than tracheobronchial washings, and the reason proposed was that the larynx is colonized earlier than the lungs. Figure 6 shows the anatomical area from which laryngeal swabs are collected. Concerning the oral fluids, their use still needs to be optimized as it has been shown that over time the pathogen was not consistently detected in pens that previously tested positive (Hernandez-Garcia *et al*. 2017).

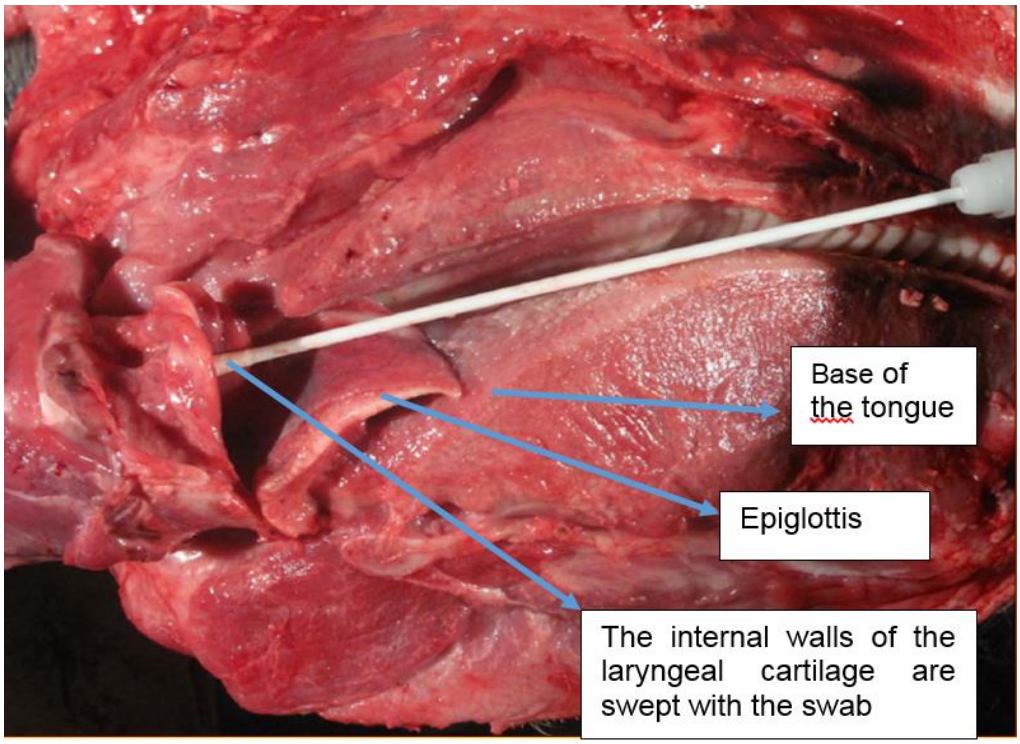



**Figure 6.** Sagittal section of the oropharynx and the nasopharynx of a pig, showing the area to sample for a laryngeal swab (picture obtained from the following link: https://www.pig333.com/articles/replacements-adaptation-in-mycoplasma-hyopneumoniae-control_11200/).

## 1.6. TREATMENT AND CONTROL OF *MYCOPLASMA HYOPNEUMONIAE* INFECTIONS

Control of *M. hyopneumoniae* infections can be achieved by combining several interventions, such as the optimization of the management practices and microclimate conditions in the different production units, and the use of antimicrobial medication and vaccination (Thacker and Minion, 2012).

### 1.6.1. Optimizing management practices and microclimate conditions

The optimization of the management practices and microclimate conditions in the production units should take into account some of the risk factors for the occurrence and transmission of *M. hyopneumoniae* that are mentioned in Table 2 (subsection **1.3.3. Factors influencing the dynamics of infection**). The most important of these factors are the implementation of a strict all-in/all-out system across all production stages and a purchase policy that considers the infection status of both the donor and recipient production site (Maes *et al*., 2008).

By avoiding mixing young age groups with older age groups, the horizontal transmission of the pathogen can be reduced, thus allowing the herd veterinarian to focus his interventions on the production phase where the majority of the animals become infected. An additional advantage of all-in/all-out production is that farmers can maintain the most suitable microclimate conditions for each age group, since as mentioned in Table 2 low ventilation rates, non-optimal indoor temperatures and/or high air humidity should be avoided (Flesjå *et al*., 1982; Done *et al*., 1991; Stärk *et al*., 2000). All-in/all-out production also allows to clean and disinfect the different units prior to the introduction of new animals. This is important as it contributes to the reduction of transmission of other respiratory infections between different batches, such as PRRSV, PCV-2 and *A. pleuropneumoniae*, where *M. hyopneumoniae* can have a potentiating or additive effect (Van Alstine, 2012).

In herds where the piglets are found to be infected with *M. hyopneumoniae* already from the peri-weaning period, it may be that the choice of a four-week batch production system over a three-week batch production system can help to reduce the vertical transmission of the pathogen to the piglets (Nathues *et al*., 2014). The reason is that four-week batch systems allow the production of larger batches, where most of the piglets present at the farrowing house are of the same age and also, they do not allow the use of foster sows. Additionally, piglets are weaned at three weeks of age, compared to three-week batch systems where piglets remain longer with the sows, i.e. until four weeks of age (Vermeulen *et al*., 2016). However, it should be mentioned that the choice of the batch production system depends a lot on the legislations effective in each place, and also on the herd characteristics (i.e. the spatial allocation of the different units and the ability of the farmer to perform an efficient artificial insemination on larger batches of animals).

Last but not least, the purchase policies of the replacement gilts constitute one of the highest risks for herds to become infected (i.e. *M. hyopneumoniae*-free SPF herds) or re-infected (i.e. endemically infected herds). High sow replacement rates or the purchase of gilts from multiple sources of unknown infection status should be avoided. Concerning the latter point, it is important to perform adequate diagnostics and match the infection status of the rearing site with the infection status of the recipient site. Table 4 (section **1.5. Diagnosis**) can help in this approach, by providing a guideline (Galina and Clavijo, 2016; Garza-Moreno *et al*., 2018).

The introduction of naive (negative or provisionally negative for *M. hyopneumoniae*) gilts into a population of subclinically or clinically infected sows brings several challenges (Maes *et al*., 2017). The reason is that this practice can destabilize the immunity of both the incoming gilts as well as the sows already existing in the recipient site. Naïve gilts can be infected upon contact with the positive sows in the recipient site. These gilts can further transmit the pathogen to other

sows in the recipient site that are possible negative. As a result, a vicious cycle is created, where the vertical transmission of the pathogen to the piglets is constantly maintained.

In these cases, it is important to create a uniform infection and immune status, which will reduce the circulation of the pathogen (Nathues *et al*., 2013a; Galina and Clavijo, 2016; Garza-Moreno *et al*., 2018). This can be achieved by the combination of the following strategies: a) placing the replacement gilts in the quarantine unit as early as possible (in terms of age), b) vaccinating them against *M. hyopneumoniae*, c) make sure that the gilts purchased are already exposed to *M. hyopneumoniae* from a young age (approximately 50 days of age). The latter strategy aims to minimize the shedding of the pathogen from the primiparous sows to their piglets at first farrowing. Previous research has shown that infected pigs can shed the pathogen to naïve pigs for a period up to 214 days after becoming infected (Pieters *et al*., 2009). Thus, taking into account that first farrowing can occur between 320 and 350 days of age, primiparous sows will have already recovered by that time and shedding would ideally be non-existent (Pieters and Fano, 2016). Nevertheless, the purchase of gilts that are already exposed at a young age poses the risk of introducing new *M. hyopneumoniae* strains of unknown virulence in the herd.

### 1.6.2. Antimicrobial medication

Although antimicrobial medication is capable of reducing clinical signs and lung lesions, together with reducing the impact of pathogens that are secondarily involved in PEP, it does not achieve complete elimination of the pathogen from the respiratory tract (Thacker and Minion, 2012). Additionally, clinical outbreaks can still be observed after medication has ceased. Nowadays, in several countries there are intensive efforts to reduce antimicrobial usage, due to the occurrence of antimicrobial resistance in different bacterial pathogens (Postma *et al*., 2015). Thus, vaccination, and the optimization of the management practices and microclimate conditions have gained significance.

***Antimicrobials available***

There is a wide variety of antimicrobials available for controlling *M. hyopneumoniae* infections. The most frequently used ones are tetracyclines and macrolides (Maes *et al*., 2017). Other antimicrobials that are potentially active against *M. hyopneumoniae* are aminoglycosides and aminocyclitols, florfenicol, fluoroquinolones, lincosamides and pleuromutilins. Among the aforementioned, only fluoroquinolones and aminoglycosides have a mycoplasmacidal effect. It should be mentioned that beta-lactam antibiotics (e.g. penicillin, amoxicillin, cefquinome and ceftiofur) are not active against *M. hyopneumoniae*, since the pathogen lacks a cell wall (Thacker and Minion, 2012). Mycoplasmas are also intrinsically resistant against (potentiated) sulfonamides and polymyxins.

Tetracyclines such as doxycycline and oxytetracycline are commonly used under field conditions. They can be administered *via* the feed or the water, and they have a broad spectrum of action against Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001). Apart from being active against *M. hyopneumoniae* (Del Pozo Sacristán *et al*., 2012a), they are also active against bacteria that can be secondarily involved in PEP, such as *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* (De la Fuente *et al*., 2007; De Jong *et al*., 2014). Nevertheless, variable degrees of acquired antimicrobial resistance towards tetracyclines has been described for *H. parasuis*, *P. multocida* and *S. suis*. Therefore, in complicated cases of PEP, antimicrobial susceptibility testing is advised to support antimicrobial therapy (Aarestrup *et al*., 2008; Dayao *et al*., 2014).

Macrolides such as gamithromycin, tildipirosin, tilmicosin, tulathromycin and tylvalosin can be used, usually intramuscularly (i.m.) or *via* the feed. Their advantage is that they can reach high therapeutic concentrations in most of the tissues, due to their high liposolubility (Pyörälä *et al*., 2014). Apart from being active against *M. hyopneumoniae*, they can also treat infections caused

by *A. pleuropneumoniae, B. bronchiseptica, H. parasuis*, *S. suis* and *P. multocida*, provided no acquired resistance is interfering with therapeutic success.

Aminoglycosides (e.g. apramycin, gentamicin, paromomycin and neomycin) and aminocyclitols (spectinomycin) are used either i.m., or *via* the feed or water. This class of antimicrobials poses the limitation that after oral administration they are poorly absorbed (Giroux *et al*., 1995). Thus, their oral use is destined for the treatment of intestinal diseases only. However, when administered i.m. they can easily penetrate the lung parenchyma and the bronchial secretions (Goldstein *et al*., 2002).

Florfenicol can be administered i.m. or *via* the water. The advantage of this antimicrobial is that it can be absorbed and distributed relatively quickly. Although controversial results have been observed for the treatment of *M. hyopneumoniae* infections (Del Pozo Sacristán *et al*., 2012b), it is very suitable for pathogens secondarily involved in PEP (i.e. *A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis* and *P. multocida*; Shin *et al*., 2005).

Fluoroquinolones (e.g. enrofloxacin and marbofloxacin) can be administered i.m. or *via* the water. They have a broad spectrum of action against aerobic Gram-positive and Gram-negative bacteria. Additionally, they achieve a high tissue concentration (Martinez *et al*., 2006). Thus, they can be utilized for both *M. hyopneumoniae* and other pathogens that are secondarily involved in PEP. However, in many countries they are not advised to be used as a first or second choice, due to the efforts to reduce the spread of antimicrobial resitance to other pathogens (Ungemach *et al*., 2006). Concering lincosamides (lincomycin), products that combine lincomycin and spectinomycin (administered i.m., or via the feed or water) have been reported to be of some value for the treatment of PEP, nevertheless they are less efficacious than tetracyclines, macrolides and fluoroquinolones (Stipkovic *et al*., 2001). Pleuromutilins (i.e. tiamulin and valenmulin, administered i.m. or *via* the feed) are not advised to be used for the treatment of PEP, although

they reach high concentrations in the lung (Zhang *et al*., 2011). The reason is that they are reserved for the treatment of Brachyspira intestinal disease (Massacci *et al*., 2018).

***Administration strategies***

In herds that present recurrent episodes of PEP in their fattening pigs, antimicrobial medication can be used strategically to approximately one week prior to the expected onset of respiratory distress (Maes *et al*., 1996; 2008). Depending on the product used, treatments can last between one and three weeks. Antimicrobials can also be used in later production phases, in an intermittent way. Nevertheless, the use of antimicrobial agents will contribute to the selection and spread of antimicrobial resistance in both *M. hyopneumoniae* and other bacterial organisms present on the farm.

### 1.6.3. Vaccination

Vaccination against *M. hyopneumoniae* is commonly practiced worldwide, with a wide range of commercial vaccines being available (Table 5). The vast majority of these vaccines are bacterins, consisting of inactivated, adjuvanted whole-cell preparations (Maes *et al*., 2017).

Vaccination has been shown to improve daily weight gain and feed conversion ratios (Maes *et al*., 2008), and also to reduce mortality rates. Additionally, milder clinical signs are observed, together with a reduction in the prevalence and severity of macroscopic *Mycoplasma*-like lung lesions. All these beneficial effects are also associated with a reduction in antimicrobial usage in the vaccinated herds (Maes *et al*., 1999). Last but not least, it has been shown that vaccination is able to reduce the load of *M. hyopneumoniae* organisms in the respiratory tract and the proportion of animals infected (Sibila *et al*., 2007b; Meyns *et al*., 2006; Vranckx *et al*., 2012b). Nevertheless, vaccination alone is not able to prevent colonization of pigs or eliminate the pathogen from the infected herds (Maes *et al*., 1998; Wilson *et al*., 2012; Del Pozo Sacristán *et al*., 2014).

**Table 5**. Most commonly used commercial vaccines against *M. hyopneumoniae* (table obtained from Maes *et al*., 2017).

| Vaccine | Antigen/strain | Adjuvant | Route of administration | Age of administration (days) | Boost needed after ...weeks |
|---|---|---|---|---|---|
| Hyogen (Ceva) | Ceva strain BA 2940-99 | Imuvant (W/O J5 LPS) | IM | ≥21 | — |
| HYORESP (Merial) | NI[a] | Aluminium hydroxide | IM | ≥5 | 3–4 |
| INGELVAC MYCOFLEX (Boehringer Ingelheim) | J strain isolate B-3745 | Impran (water-in-oil adjuvant emulsion) | IM | ≥21 | — |
| M+Pac (Intervet Int.)[b] | NI[a] | Mineral oil and Aluminium hydroxide | IM | ≥7 | 3–4 |
| MYPRAVAC SUIS (Hipra Lab) | J strain | Levamisole and carbomer | IM | ≥7–10 | 3 |
| PORCILIS M. HYO (Intervet) | Strain 11 | dl-α-tocopherol acetate | IM | ≥7 | 3 |
| Porcilis PCV M. HYO (MSD-Intervet Int)[c] | J Strain | Mineral oil and Aluminium hydroxide | IM | ≥21 | — |
| Porcilis MHYO ID Once (MSD-Intervet Int.) | Strain 11 | Paraffin oil and dl-α-tocoferylacetaat | ID | ≥14 | — |
| STELLAMUNE MYCOPLASMA (Eli Lilly) | NL 1042 | Mineral oil and lecithin | IM | ≥3 | 2–4 |
| STELLAMUNE ONE (Eli Lilly) | NL 1042 | Amphigen Base, and Drakeol 5 (mineral oil) | IM | ≥3 | — |
| SUVAXYN M.HYO[d] (Zoetis) | P-5722-3 | Carbopol | IM | ≥7 | 2 |
| SUVAXYN MH-ONE[e] (Zoetis) | P-5722-3 | Carbopol and squalane | IM | ≥7 | — |
| SUVAXYN M.HYO—PARASUIS[f] (Zoetis) | P-5722-3 | Carbopol and squalane | IM | ≥7 | 2 |

[a]No information available.
[b]Vaccination scheme when 1 ml is used for each administration. No boost vaccination needed if a 2 ml dose is used the first time.
[c]Combination vaccine with Porcine Circovirus type 2.
[d]Named Suvaxyn RespiFend MH in USA.
[e]Same name is used in the USA, but Amphigen is used as adjuvant in the USA, and vaccine can be administered from day one of age onwards.
[f]Combination vaccine with *Haemophilus parasuis*—named Suvaxyn RespiFend MH HPS in USA.

It should be noted, that there are herds where vaccination does not confer the expected results. This may be attributed to the co-circulation of other respiratory pathogens that participate in PRDC (see **subsection 1.4.3. Porcine respiratory disease complex**), that may also have a large impact on the development of respiratory disease compared to *M. hyopneumoniae*. Other factors that can lead to insufficient vaccination effects are (Chase and Lunney, 2012; Vangroenweghe, 2017): a) improper vaccine storage and/or technique of administration, b) timing of administration that does not take into account the dynamics of *M. hyopneumoniae* infections in the herd, and c) the virulence of the *M. hyopneumoniae* strains circulating in the herd.

The mechanisms of protection after vaccination are not yet fully elucidated. It has been implied that systemic cell-mediated immune responses are largely involved (Marchioro *et al*., 2013). Additionally, a lower infiltration of macrophages in the lung parenchyma of vaccinated pigs after infection with *M. hyopneumoniae* compared to non-vaccinated pigs has been observed (Vranckx *et al*., 2012a). So far, there is no substantial evidence on whether the presence of maternally derived antibodies interferes with protection from vaccination, thus this area still needs to be elucidated. Nevertheless, Martelli *et al*. (2006) found that vaccine-induced maternally derived antibodies did not interfere with the development of systemic IgG antibody response in piglets vaccinated at 14 days of age. Also, several products are registered nowadays for being used in piglets seven days old or younger (Table 5), and several studies have shown that such early administration is efficiacious (Reynolds *et al*., 2009; Wilson *et al*., 2012; Del Pozo Sacristán *et al*., 2014).

The vaccination strategies applied should rely on the type of herd, the dynamics of infection across the different age groups, the management practices inherent to each herd and the preferences of the farmer (Maes *et al*., 2003). Vaccination is performed with one- or two- dose schemes (Table 5). Double vaccination can be applied at various time periods, such as at one and

four weeks of age or at four and seven weeks of age. In general, one-shot vaccinations are preferred over two-shot vaccinations, due to the fact that they confer similar protection, and are less labour intensive (Roof *et al*., 2001; Alexopoulos *et al*., 2004; Greiner *et al*., 2011). In herds that face early *M. hyopneumoniae* infections, early vaccination schemes are preferred (i.e. animals are vaccinated during the suckling period, at weaning or shortly after weaning). The purpose of these protocols is to induce immunity and protection against *M. hyopneumoniae* prior to the mid of the nursery period (Calsamigila and Pijoan, 2000; Ruiz *et al*., 2003; Fano *et al*., 2007). In herds that face late *M. hyopneumoniae* infections, the vaccinations need to be transferred at later periods, namely between five and ten weeks of age.

Apart from fattening pigs some herds also chose to vaccinate the gilts (pre-pubertal breeding sows) during the quarantine period, in order to assist their acclimatization and to protect them from developing clinical signs upon being introduced into the general breeding sow population (Sibila *et al*., 2008). Nevertheless, in commercial pig herds, breeding sows during gestation are rarely vaccinated against *M. hyopneumoniae* (Bargen, 2004; Sibila *et al*., 2008). The effect of vaccination of sows on transmission of *M. hyopneumoniae* to the piglets has not yet been elucidated, as only few studies have addressed this issue (Ruiz *et al*., 2003; Sibila *et al*., 2008).

Under field conditions, two routes of vaccine administration exist: either i.m. or intradermally (Table 5). Intradermal vaccination is considered to offer the advantage that no needles are necessary, thus reducing the potential of spreading other pathogens, such as PRRSV and *Mycoplasma suis* (Beffort *et al*., 2017). Moreover, it is less painful for the animals and safer for the farmers as needle stick injuries are avoided. Several studies have shown that intradermal vaccination can offer better efficacy than i.m. vaccination in terms of the reduction of clinical signs and macroscopic *Mycoplasma*-like lung lesions (Tassis *et al*., 2012; Beffort *et al*., 2017).

Finally, during the last years there has been an increase in the use of combination vaccines. These are mostly one-dose vaccines that allow the simultaneous immunization of the animals against *M. hyopneumoniae* and one (mainly PCV-2) or two (PCV-2 and PRRSV) other pathogens. Experimental and field efficacy trials have shown that combination vaccines are equally effective in reducing clinical signs and macroscopic *Mycoplasma*-like lung lesions (Drexler *et al*., 2010; Corrégé *et al*., 2012; Herbich *et al*., 2013; Kaalberg *et al*., 2017).

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# Aims of the study

## AIMS OF THE STUDY

*M. hyopneumoniae* is the primary agent of enzootic pneumonia, a chronic respiratory disease which affects mainly grow-finishing pigs and inflicts major economic losses in the pig industry. One of the ways frequently used to control *M. hyopneumoniae* infections worldwide is vaccination. The most common vaccination strategy in practice is vaccination of the piglets during suckling or at weaning. Weaning is one of the most stressful events that pigs have to endure during their lifetime. Currently, it is not known whether the efficacy of vaccinating against *M. hyopneumoniae* can be influenced by the weaning process when vaccination is applied at the day of weaning.

Concerning the breeding sow population, vaccination of gestating sows against *M. hyopneumoniae* is not frequently practiced under field conditions. Nevertheless, breeding sows are responsible for maintaining *M. hyopneumoniae* infections within the herds and the percentage of piglets colonized with *M. hyopneumoniae* at weaning may be indicative of the number of sows shedding the pathogen during the suckling period. Thus, it is interesting to investigate whether vaccinating sows during gestation could decrease the percentage of their offspring that is colonized with *M. hyopneumoniae* at weaning as well as in the nursery units when piglets from different sows are mixed together.

The general aim of this thesis is to investigate different vaccination strategies against *M. hyopneumoniae* infections in order to improve the control of enzootic pneumonia.

The specific objectives are; 1) to assess the efficacy of vaccinating against *M. hyopneumoniae* with a one-shot commercial bacterin either at weaning or three days before weaning in pigs exposed to *M. hyopneumoniae* either under experimental or field conditions, and 2) to investigate whether *M. hyopneumoniae* vaccination of sows at the end of gestation can influence the *M.*

*hyopneumoniae* colonization status of their piglets during the peri-weaning period as well as the prevalence and extent of *Mycoplasma*-like pneumonia lesions at slaughter.

# Studies conducted

## 3.1. Efficacy of *Mycoplasma hyopneumoniae* vaccination before and at weaning against experimental challenge infection in pigs

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**Abstract**

Commercial bacterins are widely used to control *Mycoplasma hyopneumoniae* infections in pigs. However, it is not known whether the efficacy of vaccinating against *M. hyopneumoniae* can be influenced by the weaning process when vaccination is applied at the day of weaning. The present study assessed the efficacy of a single *M. hyopneumoniae* vaccination (Ingelvac MycoFLEX®) three days before weaning (V1) or at weaning (V2) against experimental challenge infection. Four weeks after vaccination, groups V1 and V2 ($n$=20 pigs each) and a non-vaccinated, positive control group (PCG) ($n$=20) were endotracheally inoculated with a virulent *M. hyopneumoniae* field strain. Five pigs were used as a negative control group. All pigs were euthanized five weeks after challenge. The main parameters investigated included macroscopic and histopathological lung lesions at necropsy, immunofluorescence (IF) staining and quantitative real-time PCR (qPCR) on broncho-alveolar lavage (BAL) fluid for quantifying *M. hyopneumoniae*. The average macroscopic lung lesion scores in groups V1, V2 and PCG were 0.54, 0.88 and 1.04, respectively ($P>0.05$). The average lymphohistiocytic infiltration scores in groups V1, V2 and PCG were 2.95, 3.16 and 3.61, respectively ($P<0.05$). The average IF scores were: V1=1.13, V2=1.19 and PCG=1.25 ($P>0.05$), the qPCR values were: V1=$10^{2.94}$, V2=$10^{2.76}$ and PCG=$10^{3.23}$ ($P>0.05$). Both vaccinated groups had lower numbers of macroscopic and histopathological lung lesions, and lower numbers of *M. hyopneumoniae* organisms in the BAL fluid compared to the PCG. However, no firm conclusions could be made on whether weaning negatively influences the efficacy of *M. hyopneumoniae* vaccination, since significant differences between the treatment groups were only obtained for the histopathological lung lesions. This could be attributed to the milder macroscopic lung lesions induced to the inoculated pigs, when compared to previous trials conducted by the same group.

**Keywords**: Weaning; *Mycoplasma hyopneumoniae*; Vaccination; Efficacy; Strain.

## Introduction

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the etiological agent of porcine enzootic pneumonia, a chronic respiratory disease affecting mainly grow-finishing pigs (Vicca *et al*., 2002; Sibila *et al*., 2008). *M. hyopneumoniae* infections have been detected in almost all countries with intensive production systems and are responsible for major economic losses in the pig industry (Vicca *et al*., 2002; Thacker *et al*., 2006). These economic losses are due to pig growth retardation, higher feed conversion ratios, increased antimicrobial use and increased susceptibility to other respiratory pathogens such as *Pasteurella multocida* (*P. multocida*), *Actinobacillus pleuropneumoniae*, *Trueperella pyogenes* (*T. pyogenes*) and *Streptococcus suis* (*S. suis*) (Maes *et al*., 2008). *M. hyopneumoniae* is also an important pathogen involved in the porcine respiratory disease complex (PRDC; Fablet *et al*., 2011; Del Pozo Sacristán *et al*., 2012a).

Control of *M. hyopneumoniae* infections can be achieved in a number of ways, such as optimizing management and housing conditions, antimicrobial medication and vaccination. In many countries, more than 70% of pig herds are vaccinated against *M. hyopneumoniae* in an effort to control the disease (Villarreal *et al*., 2010). Vaccination with commercial bacterins has been extensively proven to reduce performance losses, the severity of clinical signs and lung lesions (Maes *et al*., 1998, 1999; Jensen *et al*., 2002; Wilson *et al*., 2012; Del Pozo Sacristán *et al*., 2013). Different vaccination schemes can be implemented, depending on the type of herd, the production system, the infection pattern and the preference of the farmer.

Currently, both double and single vaccination strategies are widely practiced. Single vaccination is often administered either at one week of age or at weaning. Vaccination is often done at weaning because the piglets are handled then anyway, so vaccination can easily be included in routine farm management practices (Alacron *et al*., 2013; Del Pozo Sacristán *et al*., 2013). On the other hand, weaning is one of the most stressful events in the pig's life (Pié *et al*., 2004; Campbell *et al*.,

2013). The piglets are then experiencing the abrupt separation from the sow, they are being handled, they are being moved to other facilities, they are usually being mixed with other pigs, and they are receiving a different type of feed (Campbell *et al*., 2013; Pié *et al*., 2004;). Stress may interfere with an optimal response to vaccination, and therefore it is generally recommended not to vaccinate animals that are severely stressed (Chase *et al*., 2012).

Consequently, one important question that remains to be answered, and whose answer could contribute to the optimization of existing vaccination schemes, is whether the efficacy of vaccinating against *M. hyopneumoniae* can be influenced by the weaning process when vaccination is applied at the day of weaning. The objective of this trial was to investigate the efficacy of a one-shot vaccination applied either at weaning or three days before weaning in pigs experimentally infected with a virulent *M. hyopneumoniae* field strain.

## Methods

### Animals

Sixty-five cross-bred piglets (Topigs 20 sows and Pietrain boars) were purchased from a herd that was free of *M. hyopneumoniae* and PRRSV. Sows and fattening pigs of different age groups in the herd had been repeatedly monitored clinically and serologically, and then by means of macroscopic pneumonia lung lesions at slaughter, and no evidence for the presence of *M. hyopneumoniae* or PRRSV in the herd was found. At three weeks of age (day 21), the piglets were weaned and transported for one-half to two hours to the animal facilities of the Faculty of Veterinary Medicine, Ghent University, Belgium. From this point onwards, all the pigs received *ad libitum* a commercial, antibiotic-free feed.

### Experimental design and *M. hyopneumoniae* inoculation

At 14 days of age, prior to purchase, piglets from 20 different litters were individually ear-tagged and randomly allocated to four different groups. The three main treatment groups were: V1 (vaccinated before weaning, at 18 days of age and while still on the farm; *n*=20), V2 (vaccinated during weaning, at 21 days of age and before being transported to the experimental facilities; *n*=20) and a non-vaccinated positive control group (PCG; *n*=20). Five pigs were used as a negative control group, to verify that they remained free of *M. hyopneumoniae*. They were not vaccinated. The pigs in groups V1 and V2 received a single-shot intramuscular (i.m.) injection (1 mL) of a commercial *M. hyopneumoniae* bacterin vaccine (Ingelvac MycoFLEX®, Boehringer Ingelheim Vetmedica). The pigs in PCG were left untreated and were not injected with a placebo to simulate field conditions. Upon arrival at the Faculty of Veterinary Medicine, each group was housed in a different experimental room equipped with a high efficiency particulate air filter to avoid possible transmission of the pathogen between groups.

At 48 days of age, the two vaccinated groups, V1 and V2, as well as the PCG were challenge infected endotracheally with a 7 mL inoculum containing $10^7$ color changing units per mL of the virulent *M. hyopneumoniae* F7.2C field strain as described previously (Vicca *et al*., 2003). The pigs in the negative control group were inoculated with 7 mL of sterile culture medium. For the inoculations, the pigs were anaesthetized by administering a mixture of Xyl-M® 2% and Zoletil 100® i.m. at a dose rate of 0.3 mL/kg bodyweight.

All animals were euthanized and necropsied at 83 days of age. Deep anesthesia was induced by administering i.m. 0.3 mL/kg of a mixture of Xyl-M® 2% and Zoletil 100®, followed by exsanguination. The study was approved by the ethical committee for animal experiments of the Faculty of Veterinary Medicine, Ghent University (EC2013/35).

**Parameters of comparison**

Clinical and performance parameters

Each treatment group was examined daily for a minimum of 15 min for the presence of clinical signs throughout the entire trial period. Coughing severity was evaluated in a blinded manner by means of a respiratory disease score (RDS) on a scale from 0 to 6 (Halbur *et al*., 1996). According to that scale: 0 (no coughing), 1 (mild coughing after being encouraged to move), 2 (mild coughing while at rest), 3 (moderate coughing after being encouraged to move), 4 (moderate coughing while at rest), 5 (severe coughing after being encouraged to move), and 6 (severe coughing while at rest). The daily RDS values were averaged for the three following periods: from day 29 until day 47, from day 48 (day of inoculation) until day 83 (day of necropsy), and from day 29 until day 83.

Individual bodyweights were measured at 20, 48 and 83 days of age to determine the average daily weight gain (ADG) of each treatment group during the three following periods: from day 20 until day 48, from day 48 until day 83 and from day 20 until day 83.

Macroscopic and histopathological lung lesions, and immunofluorescence (IF) testing for *M. hyopneumoniae*

After necropsy, *Mycoplasma*-like macroscopic lung lesions were quantified in a blinded manner using a lung lesion score diagram (Hannan *et al*., 1982). Theoretically, the total lung lesion scores could range between 0 (absence of lesions) and 35 (entire lung affected).

Two lung tissue samples per lobe (apical, cardiac and diaphragmatic) were collected from the right lung of each pig for histopathology and IF testing. Samples were collected from the edges of the lung lesions, if present (Villarreal *et al*., 2012).

For histopathology, the tissue was fixed in 10% neutral buffered formalin and routinely processed and embedded in paraffin. One slide was made per lobe sample. Using light microscopy, the severity of peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation consistent with *M. hyopneumoniae* lesions were scored on a scale from 1 to 5 (Morris *et al*., 1995). Scores 1 and 2 were classified as lesions not related to *Mycoplasma* infections. Scores 3, 4 and 5 were considered to be associated with *Mycoplasma* infections (mild, moderate and severe lesions characteristic of broncho-interstitial (cuffing) pneumonia, surrounding the bronchioles but extending to the interstitium, with lymphofollicular infiltration and mixed inflammatory cell exudates). After three lobes per pig were investigated, the average score per treatment group was calculated.

The percentage of lung area occupied by air (percentage air) was examined by means of an automatic image analysis system (Optimas® 6.5, Media Cybernetics, Silver Spring, USA). This percentage is inversely proportional to the severity of peribronchiolar and perivascular lymphohistiocytic infiltration in the lung tissue and the amount of intrabronchiolar – intrabronchial exudate. An average percentage per treatment group was calculated (Vicca *et al*., 2003).

A direct IF assay was performed to assess the presence of *M. hyopneumoniae* in the lungs. The scores given could range from 0 to 3: 0 (no IF), 1 (limited IF), 2 (moderate IF), and 3 (intense IF) (Kobisch *et al*., 1978).

qPCR and bacteriological examination

During necropsy, broncho-alveolar lavage (BAL) fluid was collected from the left part of the lungs before the tissue samples for histopathology and IF were taken (Villarreal *et al*., 2009). The recovered fluid was divided into two aliquots, immediately cooled to 4 ºC, and then stored at -70 ºC until analysis. The first aliquot was used to quantify *M. hyopneumoniae* organisms by qPCR (Marois *et al*., 2010). DNA was extracted (Qiagen, Blood and tissue kit, Belgium) and qPCR was performed using the CFX96 real-time PCR detection system (Bio-Rad). The analysis was done in a double-replicate single setting. To convert the threshold values to the number of organisms, a tenfold dilution series of *M. hyopneumoniae* DNA was used. Values below the last dilution were considered as negative.

The second aliquot of BAL fluid was used for routine bacteriological culture to detect the presence of *Actinobacillus and Haemophilus* spp., and other pathogens which may affect the respiratory tract, such as *S. suis*, *B. bronchiseptica* (*B. bronchiseptica*), *P. multocida* and *T. pyogenes* (Villarreal *et al*., 2012).

Serology for *M. hyopneumoniae*

Blood samples were collected from all pigs at 21, 48 and 83 days of age to measure antibodies against *M. hyopneumoniae*, using a blocking ELISA (IDEIA, *M hyopneumoniae* EIA kit, Oxoid, UK; Del Pozo Sacristán et al., 2013). Sera with optical density (OD) values < 50% of the $OD_{buffer-control}$ were considered positive, while sera with OD values ≥ 50% of the $OD_{buffer-control}$ were considered negative.

**Statistical analysis**

The statistical analysis was performed on groups V1, V2 and PCG. Pigs of the negative control group (*n*=5) were not included, as they were only employed to ensure that the animals remained free of *M. hyopneumoniae* throughout the experiment. The RDS data were analyzed using repeated measures analysis of variance (ANOVA). Levene's test was used to assess the homogeneity of the variances between the different groups. The ADG, macroscopic and histopathological lung lesions, percentage of air in the lungs, IF testing and qPCR were analyzed with a non-parametric Kruskal-Wallis. These five continuous variables did not fulfill the criteria for homogeneity of variances. Statistical results were considered significant when the P-values were $\leq 0.05$ (two-sided test). The statistical package SPSS 21.0 for Windows (SPSS 21, SPSS Inc., IL, USA) was used to analyze the data.

## Results

Clinical and performance parameters

In total, two pigs died during the trial, after receiving the endotracheal challenge infection (one in the V2 group and one in the PCG group). These pigs were not included in the analysis. In groups V1, V2 and PCG, coughing started approximately six days after challenge and continued to increase towards the end of the trial. In groups V1 and V2, coughing peaked between 66 and 70 days of age, whereas in the PCG, the peak was noticed at 80 days of age. The RDS values for the period before challenge and the period after challenge, as well as for the overall period, are shown in Table 1. There were no significant differences between the groups ($P>0.05$).

The ADG results of groups V1, V2 and PCG during the period before challenge and during the period after challenge, as well as during the overall period, are presented in Table 2. There were no significant differences ($P>0.05$) between the groups.

**Table 1**. Average (± standard deviation) of the daily RDS of the pigs for the three different periods.

| Age range (days) | V1 (*n*=20) | V2 (*n*=19) | PCG (*n*=19) | P value |
|---|---|---|---|---|
| 29-47 | 0.07±0.26 | 0.28±0.69 | 0.11±0.37 | 0.108 |
| 48-83 | 0.79±1.30 | 0.62±1.20 | 0.65±1.19 | 0.626 |
| 29-83 | 0.54±0.94 | 0.50±1.03 | 0.47±0.91 | 0.852 |

Scores ranged from 0 (no coughing) to 6 (severe coughing undisturbed) (Halbur *et al*., 1996). Treatment groups: V1 (vaccinated before weaning, at 18 days of age), V2 (vaccinated on the day of weaning, at 21 days of age) and PCG (non-vaccinated). Overall and pairwise comparisons did not reveal any significant differences between groups for the three different periods. D48: experimental infection, D83: euthanasia.

**Table 2**. ADG (± standard deviation SD) of each treatment group during the three different periods.

| | ADG (±SD) (g/pig/day) | | | |
|---|---|---|---|---|
| Age range (days) | V1 (*n*=20) | V2 (*n*=19) | PCG (*n*=19) | P value |
| 20-48 | 293±41 | 299±42 | 303±55 | 0.491 |
| 48-83 | 701±116 | 681±129 | 718±140 | 0.442 |
| 20-83 | 519±56 | 511±66 | 533±71 | 0.461 |

Treatment groups: V1 (vaccinated before weaning, at 18 days of age, challenge infected), V2 (vaccinated on the day of weaning, at 21 days of age, challenge infected) and PCG (non-vaccinated, challenge infected). Overall and pairwise comparisons did not reveal any significant differences between the groups for the three different periods. D48: experimental infection, D83: euthanasia.

Macroscopic and histopathological lung lesions, and IF testing for *M. hyopneumoniae*

Only numerical differences in macroscopic lung lesions (P>0.05) were found between groups V1, V2 and PCG (Table 3). The lowest average macroscopic lung lesion score was given to group V1 (0.54). The average lung lesion scores of the pigs in groups V2 and PCG were 0.88 and 1.04, respectively. There were no macroscopic lung lesions in the negative control group.

Statistically significant differences (P<0.05) in histopathological lung lesions were present between groups V1, V2 and PCG (Table 3). The average lymphohistiocytic infiltration score was lower in group V1 (2.95) when compared with groups V2 (3.16) and PCG (3.61). The V1 group had the highest average percentage of air in the lung tissue (44.28), followed by group V2 (37.47) and PCG (31.46).

The results from the IF testing that was used to semi-quantitatively assess the load of *M. hyopneumoniae* organisms in the lung tissue, namely 1.13 (group V1), 1.19 (group V2) and 1.25

(PCG), were not significantly different (P>0.05) between groups (Table 3). All pigs of the negative control group were negative for IF staining.

qPCR assay and bacteriological examination

The number of *M. hyopneumoniae* organisms quantified by qPCR in the BAL fluid was not significantly different (P>0.05) between groups V1, V2 and PCG (Table 3). Lower numbers of organisms were detected in groups V1 and V2 ($10^{2.94}$ and $10^{2.76}$ copies/mL, respectively) compared to group PCG ($10^{3.23}$ copies/mL). No *M. hyopneumoniae* DNA was detected in pigs of the negative control group.

Several bacteria were isolated from BAL fluid. In groups V1 and V2, *Bordetella bronchiseptica* (V1: 3/20 pigs; V2: 1/20 pigs) and polybacterial cultures (V1: 4/20 pigs; V2: 2/20 pigs) were obtained. In the PCG, *Haemophilus parasuis* (1/20 pigs), *S. suis* (1/20 pigs) and polybacterial cultures (1/20 pigs) were obtained. The bacteriological culture remained negative for all pigs in the negative control group.

**Table 3**. Average (± standard deviation SD) values of macroscopic and histopathological lung lesion scores, IF scores, and qPCR.

| Parameter [a] | V1 (*n*=20) | V2 (*n*=19) | PCG (*n*=19) | P value |
|---|---|---|---|---|
| Macroscopic lung lesions | $0.54\pm0.67^{A}$ | $0.88\pm1.45^{A}$ | $1.04\pm2.45^{A}$ | 0.777 |
| Lymphohistiocytic infiltration | $2.95\pm0.50^{A}$ | $3.16\pm0.58^{B}$ | $3.61\pm0.59^{C}$ | 0.002 |
| Percentage of air in lung tissue | $44.28\pm14.68^{A}$ | $37.47\pm17.76^{B}$ | $31.46\pm14.51^{C}$ | 0.000 |
| IF | $1.13\pm0.54^{A}$ | $1.19\pm0.79^{A}$ | $1.25\pm0.70^{A}$ | 0.896 |
| qPCR on BAL fluid | $10^{2.94\pm1.24A}$ | $10^{2.76\pm1.48A}$ | $10^{3.23\pm1.33A}$ | 0.616 |

Treatment groups: V1 vaccinated before weaning, at 18 days of age, challenge infected, V2 vaccinated at weaning, at 21 days of age, challenge infected, and PCG positive control group i.e. non-vaccinated, challenge infected.

Values with different superscript (A-C) in capital letter within a row are significantly different ($P\leq0.05$).

[a] Macroscopic lung lesion scoring based on Hannan *et al*. (1982). Lymphohistiocytic infiltration score based on Morris *et al*. (1995).
Percentage of air in lung tissue measured by means of automatic image analysis system (Optimas® 6.5, Media Cybernetics, Silver Spring, USA).
IF scoring based on Kobisch *et al*. (1978).
Values from qPCR on BAL fluid expressed as log copies of *M. hyopneumoniae*/mL.

Serology

The serological results for *M. hyopneumoniae* at 21, 48 and 83 days of age are presented in Table 4. At 21 days of age, all the piglets were serologically negative for *M. hyopneumoniae*. At 48 days of age, 40% of the pigs in each of the vaccinated groups V1 and V2 had seroconverted. None of the pigs in the PCG had seroconverted to *M. hyopneumoniae* at that time. At 83 days of age, all the pigs in groups V1 and V2 were seropositive, while 95% of the pigs in the PCG had seroconverted. All pigs in the negative control group were serologically negative at all time-points.

**Table 4**. Percentage of seropositive pigs in the different groups at different time-points during the trial.

| Days of age | V1 (*n*=20) | V2 (*n*=19) | PCG (*n*=19) |
|---|---|---|---|
| 21[a] | 0 | 0 | 0 |
| 48[b] | 40 | 40 | 0 |
| 83[c] | 100 | 100 | 95 |

[a]Day of weaning and transportation to the animal facilities of the Faculty of Veterinary Medicine, Ghent University.
[b]Inoculation time.
[c]Necropsy
Treatment groups: V1 (vaccinated before weaning at 18 days of age, challenge infected), V2 (vaccinated on the day of weaning at 21 days of age, challenge infected), PCG (non-vaccinated, challenge infected) and NCG (non-vaccinated, non-challenge infected).

## Discussion

The present experimental trial investigated whether the efficacy of one-shot vaccination is influenced by the weaning process. Differences between V1 (vaccination three days before weaning), V2 (vaccination at weaning) and the PCG (no vacination) were small and mostly statistically not significant, except for the microscopic lung lesions, which were lower in V1.

Apart from the transport related to the weaning process, the piglets in the present study were also transported to the experimental facilities, a trip that lasted anywhere from one-half to two hours. In fact, age- and site-segregated pork production is a complex process that involves movement of the piglets from the farrowing house to the nursery facilities, which are situated in different locations (Whiting and Brandt, 2002). It can be expected, though, that the stress imposed on the piglets in our study may have been greater than when the piglets remain on the same site and are not transported (e.g. in single site farrow-to-finish pig herds) (Lambooij *et al*., 2012).

Vaccination at the moment of weaning, when the piglets are being handled anyway, is a common practice, as it can be implemented easily in the daily management of a pig herd. The pigs of V1 were vaccinated three days before weaning. This vaccination strategy is also commonly practiced by some pig producers in order to avoid any possible negative effects of the weaning process, and/or to avoid too much handling or interventions on the same day (Gillespie *et al*., 2010). A difference of three days between the two vaccination groups most probably allowed sufficient time for the development of the first and most critical steps of the immune response prior to the stress of weaning (Kick *et al*., 2011). At the same time, a too great difference in vaccination age was avoided between the piglets of groups V1 and V2 as a possible early disease outbreak (e.g. diarrhea) could have impacted more group V1 than group V2. Overall, the two vaccination schemes are relevant for the situation in many pig herds.

The experimental infection model used for *M. hyopneumoniae* was similar to that used in previous trials (Villarreal *et al*., 2009; 2011; 2012; Del Pozo Sacristán *et al*., 2012b). Challenge infection proved to be successful since the vast majority of the infected pigs had positive qPCR values, exhibited the presence of *Mycoplasma*-like macroscopic lung lesions at necropsy and were positive for IF staining. However, the macroscopic lung lesions observed in the present trial were milder compared to trials that had been previously conducted using the same inoculation dose of *M. hyopneumoniae* F7.2C field strain, experimental facilities and way of allocation (Meyns *et al*., 2006; Del Pozo Sacristán *et al*., 2012b; Villarreal *et al*., 2012). The precise reasons for this difference are not known. Based on the macroscopic lung lesion scores, serology, qPCR testing and IF staining, all the pigs in the negative control group were negative for *M. hyopneumoniae* throughout the trial, thus confirming that the study piglets were free of *M. hyopneumoniae*.

The RDS values in the PCG were similar to those mentioned in other experimental trials (Villarreal *et al*., 2011; 2012). Compared with the vaccinated groups V1 and V2, the only differences found in the PCG were numerical. There were no significant differences in ADG between the groups. This is mainly due to the high standard deviations observed and the limited numbers of pigs. ADG was measured, but it was not considered an important parameter for this experimental study. In vaccination trials under field conditions however, in which many more animals can be included, ADG is an important parameter.

Group V1 had the lowest numbers of macroscopic and histopathological lung lesions, and the lowest IF scores when compared to groups V2 and PCG. Nevertheless, significant differences between groups V1, V2 and PCG were only obtained for the histopathological lung lesions. This was confirmed by the results of the percentage of air parameter in the lung tissue measured by means of an automatic image analysis system. The statistically significant differences between groups V1, V2 and PCG proved that *M. hyopneumoniae* vaccination is effective in reducing lymphohistiocytic infiltration and increasing the percentage of air in the lung. This reduction of

the lymphohistiocytic infiltration scores in the vaccinated groups is in accordance with previous experimental trials (Villarreal *et al*., 2011; 2012). The less severe histopathological lesions may be due to a modulation of the immune response in vaccinated animals. It has been shown that vaccination with a bacterin against *M. hyopneumoniae* reduces the infiltration of macrophages in the lung tissue (Vranckx *et al*., 2012).

Although lower qPCR values were recorded in the vaccinated groups when compared to the PCG, it was not possible to conclude whether vaccination was associated with the reduced bacterial load in the lungs, since the differences between the groups were only numerical. However, the detection of *M. hyopneumoniae* in the BAL fluid of groups V1 and V2 confirms that vaccination alone is not able to prevent colonization of the pig's respiratory tract (Maes *et al*., 2008; Villarreal *et al*., 2011; 2012).

The pigs of groups V1, V2 and PCG were seronegative for *M. hyopneumoniae* on the day of transportation to the experimental facilities. At the time of inoculation, 40% of the pigs in each of the vaccinated groups V1 and V2 had seroconverted. This is in agreement with other studies which have reported that vaccination with commercial bacterins induces seroconversion rates ranging from 30 to 100% (Thacker *et al*., 1998; Maes *et al*., 2008). At necropsy, all the pigs in groups V1 and V2 were seropositive, while the percentage of pigs that had seroconverted in group PCG was 95%.

Overall, no firm conclusions could be made as to whether group V1 performed better than groups V2 and PCG, since only the microscopic lung lesions were significantly lower in the pigs in group V1. Further research under field conditions is necessary to elucidate the possible effect of weaning on the efficacy of one-shot *M. hyopneumoniae* vaccination. Field trials make it possible to include more animals and to study the entire course of *M. hyopneumoniae* infections up to the time of slaughter. A future field trial could possibly provide further insight on the same topic.

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## 3.2. *Mycoplasma hyopneumoniae* vaccination at or shortly before weaning under field conditions: a randomised efficacy trial

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**Abstract**

This study assessed the efficacy of two different *Mycoplasma hyopneumoniae* vaccination programs in relation to the time of weaning. Eight hundred and twenty-eight piglets were randomly divided into three groups: group V1 was vaccinated three days before weaning, group V2 at weaning (21 days of age) and group NV was left non-vaccinated. Vaccinations were performed using Ingelvac MycoFLEX®. After the nursery period, 306 pigs were allocated to fattening unit (F1) and 501 pigs to a second unit (F2). Efficacy was evaluated using performance parameters and pneumonia lesions at slaughter. Statistically significant differences were obtained in F2 where group V1 had a higher average daily weight gain compared to groups V2 and NV for the entire study period (17 and 18 g/day, respectively) and the fattening period (26 and 36 g/day, respectively) ($P<0.05$). Considering respiratory disease scores for both fattening units, group V1 was the only group where coughing severity did not increase significantly between placement and the end of the fattening period ($P>0.05$). Between groups there were no statistically significant differences for the average lung lesion scores (V1=3.44; V2=4.61; NV=4.55, $P>0.05$) and the prevalence of pneumonia (V1=35.0%; V2=38.0%; NV=41.4%, $P>0.05$). Overall, vaccination against *M. hyopneumoniae* prior to weaning provided numerically better performance than vaccination at weaning, but did not reach statistical significance. An influenza outbreak in F1 and the presence of coexisting mixed respiratory infections in both F1 and F2, could have possibly influenced the performance of both vaccinated groups across all measured parameters.

**Keywords**: Weaning; *Mycoplasma hyopneumoniae*; Vaccination; Efficacy; Field study.

## Introduction

*Mycoplasma hyopneumoniae* is the primary agent of porcine enzootic pneumonia (PEP) which affects mainly grow-finishing pigs and inflicts major economic losses on the pig industry (Thacker and Minion, 2012). Vaccination is a very common control measure which has been extensively proven to reduce performance losses, the severity of clinical signs and lung lesions (Maes *et al*., 1998; 1999; Jensen *et al*., 2002; Wilson *et al*., 2012; Del Pozo Sacristán *et al*., 2013).

Currently, vaccination at weaning is commonly practiced as the handling of the piglets is inevitable at that time (Del Pozo Sacristán *et al*., 2013; Alarcon *et al*., 2013). On the other hand, weaning is one of the most stressful events in the life of a piglet (Pié *et al*., 2004; Campbell *et al*., 2013). It is not recommended to vaccinate animals when they are severely stressed (Chase *et al*., 2012). For that reason, vaccinating the animals two to three days before weaning is also practiced by some pig producers (Gillespie *et al*., 2010).

Consequently, one important question that remains unaddressed is whether vaccination at the day of weaning has an influence on the efficacy of *M. hyopneumoniae* vaccines. A previous study investigated the efficacy of a single *M. hyopneumoniae* vaccination three days before weaning or at weaning against experimental challenge infection with a virulent *M. hyopneumoniae* field strain (Arsenakis *et al*., 2016). The results showed that the group that was vaccinated three days before weaning had the lowest macroscopic and histopathological lung lesions. A difference of three days between the two vaccination groups was chosen to allow the first and most critical steps of the immune response to develop prior to the stress of weaning (Kick *et al*., 2011). However, significant differences between the vaccinated groups were only obtained for the histopathological lung lesions. Thus, it was considered that a field study would provide further insight on the same topic, since it would permit to include more animals, to test the effect under practical conditions

with concurrent infections with other respiratory pathogens, and to investigate performance data until slaughter age.

The objective of this study was therefore to investigate the efficacy of a one-dose *M. hyopneumoniae* vaccination applied either at weaning or three days before weaning in a Belgian pig herd with mixed respiratory disease in the fattening period, where *M. hyopneumoniae* has been involved as an important pathogen. Average daily weight gain (ADG) and the severity of *Mycoplasma*-like pneumonia lesions at slaughter constituted the primary efficacy parameters.

## Materials and methods

### Herd description

The study was conducted between September 2013 and March 2014 in a two-site Belgian pig herd, operating a four-week batch production system for the sows (Table 1). At three weeks of age, the piglets were weaned and transferred to the nursery unit located on the same site. The nursery unit consisted of six compartments with 20 pens per compartment (20 pigs per pen). Each compartment had fully slatted floors, channel ventilation and a stocking density of 0.27 $m^2$/pig. At 10 weeks of age, approximately 30% of the pigs were sold, while the remaining ones were allocated to two different fattening units, in which they were kept until slaughter (27 weeks of age). The first fattening unit (F1) was within-site and consisted of three compartments of 26 pens each (13 pigs per pen). The second fattening unit (F2) was located 5 km from the sow herd, and consisted of five compartments of six pens each (13 pigs per pen) and two compartments of four pens each (14 pigs per pen). Both F1 and F2 had fully slatted floors, conventional mechanical ventilation systems (combining vent doors and ceiling fans) and a stocking density 0.75 $m^2$/pig.

One month before the onset of the study, tracheobronchial swabs were collected from 10-, 16- and 24-week-old pigs (10 for each age group) and results showed that 7/10, 5/10 and 10/10 pigs, respectively, were positive for *M. hyopneumoniae* by nested PCR (nPCR; Stärk *et al*., 1998). Blood samples were obtained from 24-week-old pigs, and 9/10 were serologically positive for *M. hyopneumoniae* (S/P values higher than 0.4) using an indirect ELISA (HerdCheck *M. hyo*, IDEXX). One hundred of those fattening pigs were examined at the slaughterhouse, and *Mycoplasma*-like lung lesions were present on 51% of the lungs. Prior to the initiation of the study, the herd did not perform any vaccination of the fattening pigs, apart from the one against boar taint (Improvac, Zoetis).

**Table 1**. Herd description and health management practices

| | |
|---|---|
| Number of sows | 450 |
| Breed of sows | Topigs 20 |
| Breed of boars for artificial insemination | Piétrain |
| Vaccination of sows | |
| PRRSV | Ingelvac PRRS MLV, Boehringer |
| Atrophic rhinitis | Porcilis AR-T, MSD |
| *Escherichia coli* | Porcilis coli, MSD |
| Parvovirus+*Erysipelothrix rhusiopathiae* | Porcilis Ery-Parvo, MSD |
| Vaccination of gilts in quarantine unit | |
| PRRSV | Ingelvac PRRS MLV, Boehringer |
| Atrophic rhinitis | Porcilis AR-T, MSD |
| *E. coli* | Porcilis coli, MSD |
| Medication in the farrowing unit | Iron (Uniferon, Pharmacosmos) at day 5 |
| | Toltrazuril per os (Baycox, Bayer) at day 5 |
| Medication in the nursery unit (day 21-70) | In-feed zinc oxide after weaning for 14 days at 2,500 ppm. |
| Medication in the fattening unit (day 70-200) | Flubendazole at 10 weeks of age via the feed (Flubenol 5% premix, Elanco Animal Health) for five days, repeated every 6 weeks |
| | Gonadotropin-releasing factor analogue vaccine (Improvac, Zoetis) against boar taint at 10 and 18 weeks of age. |

**Study population and experimental design**

In total, 828 pigs originating from one batch of sows were selected for this study. These pigs were offspring from 69 randomly selected sows. The parity distribution of the selected sows was from 1 (n=15) to 13 (n=1), with an average±SD of 2.6±1.8 parity number. Parity-two sows constituted the largest group (n=34). At 14 days of age, the pigs were individually ear-tagged and randomly allocated to one of the three treatment groups. Within each litter, an equal number of pigs (n=4) was allocated to each treatment group (blocked randomization). This experimental design was followed so that there is no difference between treatment groups in the average parity number of the sows providing the piglets. By that way, confounding factors that might influence the results could be minimized. All allocations were performed by the third author. Finally, each treatment group consisted of 276 pigs.

The three treatment groups were: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated at the day of weaning, at 21 days of age, one to three hours prior to the separation from the sow) and a non-vaccinated group (NV). Pigs in groups V1 and V2 received a single-dose intramuscular (i.m.) injection (1 mL) of a commercial *M. hyopneumoniae* bacterin vaccine (Ingelvac MycoFLEX, Boehringer Ingelheim).

In the nursery unit piglets from maximum six different litters were regrouped according to weight and sex, while upon transfer to the fattening units, the pigs from maximum two different nursery pens were regrouped according to pen size and sex. Animals housed within the same pen in the nursery and fattening units belonged to the same treatment group. Within the nursery and fattening units, the different treatment groups were allocated alternately between the pens, until each compartment was full. In the nursery unit, only two out of the six available compartments were filled with animals included in the trial. In F1 only one compartment was used, whereas in F2 all compartments were used. During the entire trial, water and commercial feed (meal) were supplied

*ad libitum* to the pigs. Feed (commercial meal), housing, management factors, sex distribution and prophylactic treatment were the same for the three groups. The study was approved by the ethical committee for animal experiments of the Faculty of Veterinary Medicine, Ghent University (EC2013/171).

**Parameters of comparison**

Performance parameters

In order to obtain the ADG (g/pig/day), all pigs were individually weighed at two weeks of age, at the end of the nursery period (10 weeks of age) and prior to slaughter (27 weeks of age). Weighing was performed in a blinded manner with respect to the treatment status by the first and second author, and animal caretakers.

Clinical parameters

Coughing severity was measured blindly by the first author every two weeks, starting from the beginning of the fattening period. All measurements throughout the fattening period were obtained following the same pen order, each time starting at 8 am, firstly from F1 and then directly moving to F2. The number of pigs coughing per pen was counted for 10 minutes. Then, a respiratory disease score (RDS) was calculated by dividing the number of pigs per pen that coughed during 10 min, by the total number of pigs in that pen, multiplied by 100 (Mateusen *et al*., 2002; Del Pozo Sacristán *et al*., 2012).

In case of respiratory or neurological signs, only treatment with amoxicillin by i.m. injection or via the drinking water was allowed. Possible respiratory disease problems were monitored by paired sera from 10 coughing pigs, obtained during the outbreak and three weeks later (referred to as pre- and post- serum samples, respectively). The sera were analysed to detect antibodies against PRRSV (HerdCheck PRRS ELISA, IDEXX), PCV-2 (IgM and IgG, Ingezim PCV2 ELISA, Ingenasa), SIV (H1N1, H1N2, H3N2, standard haemagglutination-inhibition test), *M.*

*hyopneumoniae* (HerdCheck *M. hyo*, IDEXX), *Haemophilus parasuis* (Hps OppA, Biocheck) and *A. pleuropneumoniae* (serotypes 1, 9 or 11 and serotype 2; Swinecheck APP 1,9,11 and Swinecheck APP 2, Biovet). Additionally, paired nasal swabs from the same animals were collected to monitor SIV by a real-time reverse transcription PCR (rRT-PCR; VetMAX-Gold SIV Detection Kit, Life technologies) detecting type A SIV-specific RNA (Bowman *et al*., 2015).

A subset of dead pigs was necropsied in order to assess the possible cause of death. In all cases, necropsies were combined with bacteriological culture, antimicrobial susceptibility testing and histopathology.

Serological examination

Blood samples were collected from the same 20 randomly selected pigs per group at 2, 10, 18 and 26 weeks of age to measure antibodies against *M. hyopneumoniae*, using a blocking ELISA (IDEIA, *M hyopneumoniae* EIA kit, Oxoid, UK) as previously done by Del Pozo Sacristán *et al*. (2013). In each treatment group, equal numbers of sampled pigs were distributed across fattening units. Inhibition percentages (IP) for all sera were also calculated considering the OD-value of each serum sample as well as the negative control according to Sibila *et al*. (2004). Classification of individual sera on the basis of IP values was as follows: IP < 30%, negative; IP > 50%, positive; IP > 30% and < 50%, equivocal.

Thirty blood samples (five for each group/fattening unit) were randomly selected from the blood samples collected at 26 weeks of age. These were additionally tested for the presence of antibodies to PRRSV, PCV-2, SIV, *H. parasuis* and *A. pleuropneumoniae*. The serological tests used were the same as the ones used to investigate respiratory disease diagnostics (see clinical parameters section).

Detection of *M. hyopneumoniae* using qPCR on tracheobronchial swabs

Tracheobronchial swabs (TBS) were collected from 20 pigs per group at 10, 14 and 18 weeks of age (the same 20 pigs from which blood samples were collected). Sampled pigs were endotracheally intubated with a sterile, semirigid canine urinary catheter, as described by Vangroenweghe *et al*. (2015). All samples were immediately cooled at 4 ºC, and stored at -20 ºC until analysis. The material collected by TBS was used to quantify *M. hyopneumoniae* organisms by qPCR as previously described Marois *et al*. (2010) and Del Pozo Sacristán *et al*. (2012).

Lung lesions

The prevalence of *Mycoplasma*-like pneumonia lesions, fissures and pleurisy were recorded at slaughter *via* a blinded manner by the second author with the assistance of the first author. The total area (percentage) of macroscopically visible *Mycoplasma*-like lung lesions (lung lesion score) was quantified according to the scoring system described by Morrison *et al*. (1985a) and Del Pozo Sacristán *et al*. (2012; 2013). *Mycoplasma*-like pneumonia lesions (catarrhal bronchopneumonia) were defined as red-purplish areas of cranioventral consolidation raised above the surface or at the surface of each lobe, and with a liver-like consistency (Holyoake and Callinan, 2006; Del Pozo Sacristán *et al*., 2012; 2013). Chronic *Mycoplasma*-like pneumonia lesions (fissures) were grey to purplish cranioventral scars, shrunken below the surface of the lobes, and had a more solid texture than the unaffected neighboring parenchyma (Sørensen *et al*., 2006; Del Pozo Sacristán *et al*., 2012; 2013). Pleurisy was evident as fibrous adhesions between the lung lobes and/or the lungs and thoracic wall (Del Pozo Sacristán *et al*., 2012; 2013; Michiels *et al*., 2015).

**Statistical analysis**

The number of animals in each treatment group (276) allowed to assess a difference of 19 grams (SD=80) in ADG and 3.2 points (SD=13) in lung lesion score with 95% certainty and 80% statistical power (Win Episcope 2.0, Edinburgh, UK). The ADG and lung lesion score were the

primary outcome parameters. Analysis of variance (ANOVA) was used to analyze bodyweights, ADG and RDS with treatment, compartment and sex included as fixed factor, and pen as a random variable. In the combined model (including both fattening units), unit was additionally included as a random variable. Pair-wise comparisons between the different treatment groups were made using Scheffe's test. Data that did not fulfill the criteria of normality and homogeneity of variances (lung lesion scores, qPCR values and serology IP) were analyzed using non-parametric Kruskal-Wallis. RDS and qPCR (time and group effect) data were analyzed using repeated measures ANOVA. The average RDS summarized until 20 weeks of age and between 20 and 26 weeks of age were compared within each group using paired t-tests. Mortality rate, the percentage of pigs showing *Mycoplasma*-like lesions, fissures and pleurisy, the percentage of pigs showing *M. hyopneumoniae* antibodies and the percentage positive on qPCR were analyzed using logistic regression with treatment and fattening unit as predictors for the model. Statistical results were considered significant when P-values were $\leq 0.05$ (two-sided test). The statistical package SPSS 21.0 was used to analyze the data.

## Results

Performance parameters

No statistically significant differences for the average bodyweight were shown between groups at inclusion in the study (2 weeks of age) (P=0.919), end of the nursery period (10 weeks of age) (P=0.109) and prior to slaughter (at 27 weeks of age, taking into account both fattening units) (P=0.263) (Table 2). When taking into account each fattening unit separately (Table 3), significant differences between groups for weight gains and ADG were observed only in F2. More specifically, group V1 had significantly higher weight gains and ADG compared to groups V2 and NV.

Clinical parameters

There was a significant difference between F1 and F2 in the overall average RDS (no group effect) between 20 and 26 weeks of age (P=0.011). Coughing severity increased towards the end of the fattening period (between 20 and 26 weeks of age) (Table 4). Taking into account the average of both fattening units, the paired t-test results revealed significant differences between the two fattening periods in groups NV and V2 (P=0.031 and P=0.007, respectively), but not in group V1 (P=0.194).

During both fattening periods from 10 to 20 and from 20 to 26 weeks of age, higher average RDS were observed in F1 compared to F2 (Table 4). The coughing severity in F1 increased at approximately 20 weeks of age among all groups (Figure 1). For that reason, all pigs in F1 were medicated for a five-day period with amoxicillin via the drinking water. Paired sera from F1 showed that 8/10 pigs had a positive post-serum titer (higher than 80) for the H3N2 subtype of SIV, with an average of 152. All pre-serum samples had a titer of 20. Moreover, all nasal swabs (10/10) collected at the same time as the pre-serum were positive for SIV type A. Serology for *M. hyopneumoniae* showed an average S/P ratio of 0.81 for the pre-serum (9/10 pigs positive) and an

average S/P ratio of 1.02 for the post-serum (9/10 pigs positive). Additional serology on pre- and post- serum samples showed that at both collection points 10/10 animals were positive for PRRSV. Also, 10/10 pigs and 1/10 pigs were tested PCV-2 IgG and PCV-2 IgM positive, respectively.

**Table 2**. Performance parameters from 2 to 27 weeks (w) of age and percentage of market pigs in the three groups with *Mycoplasma hyopneumoniae*-like lesions (*Mhyo*), fissures and pleurisy, and severity of lung lesions expressed as lung lesion score (average ± standard deviation SD). Average values (±SD) were calculated taking into account both fattening units (F1 and F2).

| | Age (w) | V1 | V2 | NV | P value |
|---|---|---|---|---|---|
| **Performance parameters[a]** | | ***n*=270** | ***n*=266** | ***n*=271** | |
| Average bodyweight (kg) | 2 | 4.28±0.78 | 4.32±0.77 | 4.31±0.76 | 0.919 |
| | 10 | 23.56±3.76 | 23.99±3.71 | 24.28±3.90 | 0.109 |
| | 27 | 106.81±12.96 | 105.21±13.88 | 104.12±15.17 | 0.263 |
| Weight gain (kg) | 10-27 | 83.26±11.44 | 81.46±12.41 | 80.10±13.42 | 0.094 |
| | 2-27 | 102.55±12.76 | 100.95±13.68 | 99.71±14.90 | 0.164 |
| ADG (g/pig/day) | 2-10 | 371±64 | 376±69 | 384±67 | 0.076 |
| | 10-27 | 718±98 | 701±108 | 688±116 | 0.094 |
| | 2-27 | 610±76 | 600±81 | 593±88 | 0.164 |
| **Lung lesions** | | ***n*=134** | ***n*=128** | ***n*=129** | |
| Prevalence of *Mhyo*-like lesions | market | 35.0 | 38.0 | 41.4 | 0.602 |
| Prevalence of fissures | market | 37.5 | 38.7 | 39.7 | 0.943 |
| Prevalence of pleurisy | market | 7.2 | 9.6 | 12.5 | 0.381 |
| Lung lesion score | market | 3.44±6.77 | 4.61±11.11 | 4.55±9.99 | 0.617 |

Treatment groups: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (non-vaccinated group). Differences between groups were not statistically significant (P>0.05).

[a] Average daily gain (ADG)

**Table 3**. Performance parameters between 2 and 27 weeks (w) of age and percentage of market pigs in the three groups with *Mycoplasma hyopneumoniae*-like lesions (*Mhyo*), fissures and pleurisy, and severity of lung lesions expressed as lung lesion score (average± standard deviation SD). Average values (±SD) are presented for each fattening unit (F1 and F2), separately.

| | | (F1) | | | | (F2) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Age (w) | V1 | V2 | NV | P value | V1 | V2 | NV | P value |
| **Performance parameters[a]** | | ***n*=101** | **n=102** | ***n*=103** | | ***n*=169** | ***n*=164** | ***n*=168** | |
| Average bodyweight (kg) | 27 | 98.43±11.20 | 100.10±14.05 | 98.20±13.16 | 0.152 | 111.03±11.70 | 108.13±13.00 | 108.17±15.16 | 0.065 |
| Weight gain (kg) | 10-27 | 76.12±9.30 | 77.15±12.48 | 75.25±11.64 | 0.124 | 86.80±10.74$^{A}$ | 83.96±11.69$^{B}$ | 83.25±13.60$^{B}$ | 0.011 |
| | 2-27 | 94.33±10.98 | 95.91±13.90 | 93.99±12.94 | 0.124 | 106.68±11.55$^{A}$ | 103.81±12.73$^{B}$ | 103.65±14.92$^{B}$ | 0.038 |
| ADG (g/pig/day) | 10-27 | 656±81 | 665±109 | 648±100 | 0.124 | 748±92$^{A}$ | 722±101$^{B}$ | 712±117$^{B}$ | 0.010 |
| | 2-27 | 562±65 | 570±82 | 559±77 | 0.139 | 634±68$^{A}$ | 617±76$^{B}$ | 616±88$^{B}$ | 0.034 |
| **Lung lesions** | | ***n*=39** | ***n*=37** | ***n*=39** | | ***n*=95** | ***n*=91** | ***n*=90** | |
| Prevalence of *Mhyo*-like lesions | market | 32.4 | 41.0 | 48.7 | 0.357 | 36.1 | 36.9 | 37.6 | 0.980 |
| Prevalence of fissures | market | 51.3 | 43.6 | 48.7 | 0.788 | 31.3 | 36.9 | 35.0 | 0.727 |
| Prevalence of pleurisy | market | 7.5 | 14.6 | 15.0 | 0.529 | 7.0 | 7.6 | 11.2 | 0.579 |
| Lung lesion score | market | 3.68±7.57 | 4.88±11.79 | 3.56±5.93 | 0.628 | 3.33±6.43 | 4.50±10.88 | 5.04±11.51 | 0.844 |

Treatment groups: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (non-vaccinated group). Within a row, different superscript letters indicate significant differences between values ($P<0.05$).

[a] Average daily gain (ADG)

**Table 4**. The average (± standard deviation SD) respiratory disease score (RDS) of the pigs between 10 and 20 weeks of age and between 20 and 26 weeks (w) of age. Average values (±SD) were calculated taking into account both and each fattening unit (F1 and F2) separately.

| Age range (w) | V1 | V2 | NV | P value |
|---|---|---|---|---|
| Average both units | ***n*=270** | ***n*=266** | ***n*=271** | |
| 10-20 | $1.76\pm1.90^{A}$ | $1.55\pm1.94^{A}$ | $1.77\pm1.78^{A}$ | 0.989 |
| 20-26 | $2.70\pm2.91^{A}$ | $3.68\pm2.86^{B}$ | $3.94\pm3.81^{B}$ | 0.801 |
| P* | 0.194 | 0.007 | 0.031 | |
| (F1) | ***n*=101** | ***n*=102** | ***n*=103** | |
| 10-20 | 2.22±1.98 | 2.58±2.25 | 2.25±1.40 | 0.937 |
| 20-26 | 5.45±2.70 | 5.43±2.65 | 4.08±2.99 | 0.633 |
| (F2) | ***n*=169** | ***n*=164** | ***n*=168** | |
| 10-20 | 1.55±1.91 | 1.03±1.63 | 1.55±1.94 | 0.725 |
| 20-26 | 1.43±2.05 | 2.80±2.64 | 3.87±4.25 | 0.256 |

Treatment groups: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (non-vaccinated group). Within a column, different superscript letters correspond to within group significant differences between the two fattening periods ($P^*<0.05$) (paired t-tests). The P value column indicates that there were no significant differences between groups during the two different fattening periods ($P>0.05$).

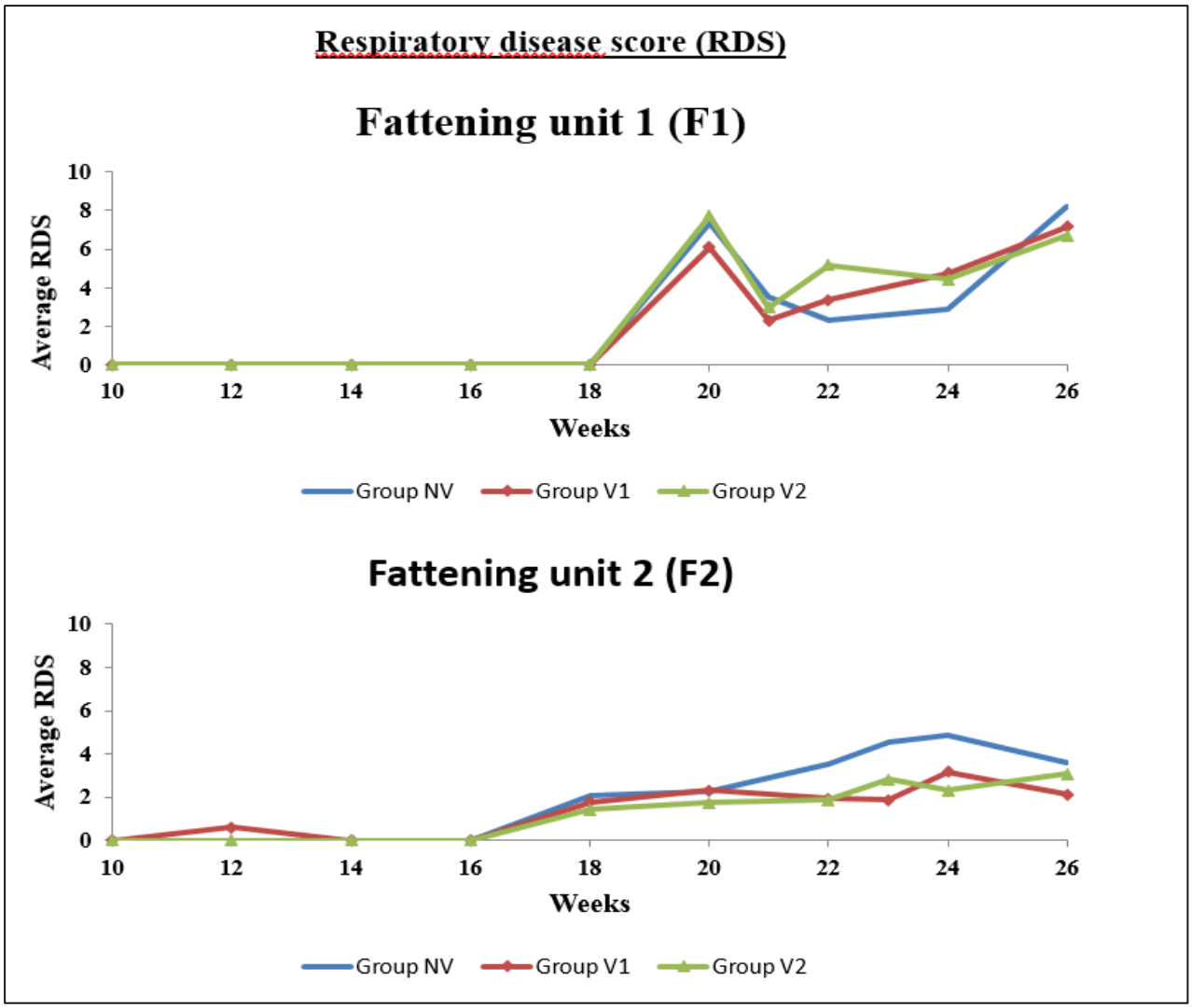


**Fig. 1**. Average respiratory disease score (RDS) over time, in each of three groups of pigs per fattening unit (F1 and F2). Average RDS, together with the standard deviations (vertical bars), from 10 weeks of age until 26 weeks of age in group V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (non-vaccinated group).

Approximately 3 weeks after the occurrence of respiratory problems in F1, sporadic non-productive coughing was also observed in F2, mainly in group NV (Figure 1). A similar five-day treatment with amoxicillin via the drinking water was used. The serological test for *M. hyopneumoniae* showed an average S/P ratio of 0.79 for the pre-serum (7/10 pigs positive) and an average S/P ratio of 1.08 for the post-serum (9/10 pigs positive). Moreover, all pre- and post- sera were positive for PRRSV and anti-PCV-2 IgG-antibodies.

The number of dead pigs in the different treatment groups are presented in Table 5. In total, 55 pigs died during the entire study period. A subset of dead animals were necropsied (17/55): 5 from the nursery and 6 each for F1 and F2. All necropsied pigs that died during the nursery period had pathological signs of fibrinopurulent pericarditis and endocarditis. *S. suis* was isolated from the heart and kidneys. The pigs that died during the fattening period showed either pathological signs of respiratory or/and wasting disease characterized by pleurisy, pericarditis and/or purulent peritonitis. Several bacteria were isolated from the lungs of those pigs; *T. pyogenes* (7/12), *P. multocida* (4/12) and *S. suis* (6/12). The main pathogen isolated from the pigs with pericarditis/purulent peritonitis was *S. suis*.

**Table 5**. Number of dead pigs per group between 2 and 27 weeks (w) of age. The contribution of each fattening unit (F1 and F2) on the combined (both units) mortality rates between 10 and 27 weeks of age is presented separately. All mortality rates were calculated according to the total number of animals included in each treatment group at the beginning of the study (n=276).

| Age range (w) | V1 | V2 | NV | P value |
|---|---|---|---|---|
| 2-10 | 6/276 (2.17) | 10/276 (3.62) | 5/276 (1.81) | 0.371 |
| 10-27 (both units) | 9/276 (3.26) | 10/276 (3.62) | 15/276 (5.43) | 0.260 |
| 2-27 | 15/276 (5.43) | 20/276 (7.24) | 20/276 (7.24) | 0.607 |
| (F1) | | | | |
| 10-27 | 3/276 (1.09) | 5/276 (1.81) | 10/276 (3.62) | 0.150 |
| (F2) | | | | |
| 10-27 | 6/276 (2.17) | 5/276 (1.81) | 5/276 (1.81) | 0.983 |

Treatment groups: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (non-vaccinated group). The P value column indicates that there were no significant differences between groups in mortality rates during the three different production periods (P>0.05).

Serological examination

The serological results for *M. hyopneumoniae* at 2, 10, 18 and 26 weeks of age are presented in Table 6. Regarding the average IP values from both units, at 10, 18 and 26 weeks of age the vaccinated groups V1 and V2 exhibited higher average values compared to NV (P=0.004, P=0.066 and P=0.159, respectively).

The serological examination for the other pathogens revealed that in F1, at 26 weeks of age, 8/15 pigs were seropositive for the H3N2 subtype of SIV, 15/15 pigs were seropositive for PRRSV and that 15/15 and 6/15 pigs were tested PCV-2 IgG and PCV-2 IgM positive, respectively. In F2, 0/15 pigs were seropositive for the H3N2 subtype of SIV, 15/15 pigs were seropositive for PRRSV, while 15/15 and 10/15 pigs were tested PCV-2 IgG and PCV-2 IgM positive, respectively.

Detection of *M. hyopneumoniae* using qPCR on tracheobronchial swabs

Group V1 had significantly lower numbers of positive animals compared to groups V2 and NV at 10 and 14 (both fattening units) weeks of age (P=0.000 and P=0.041, respectively, Table 7). The average qPCR values of *M. hyopneumoniae* organisms were lower in group V1 than in groups V2 and NV, across all sampling points, apart from F1 at 18 weeks of age where group V1 had the second lowest qPCR value (2.03) compared to groups V2 (1.78) and NV (2.28). Significant differences between groups were observed at 10, 14 and 18 (both fattening units) weeks of age (P=0.000, P=0.000 and P=0.039, respectively) (Table 7).

Lung lesions

Lung lesion evaluation at slaughter was performed on 391 pigs (n=134 V1; n=128 V2; n=129 NV). Some pigs could not be evaluated because of lost ear tags and lungs that did not reach the examination stand. Taking into account both F1 and F2 (Table 2), no statistically significant differences were found between treatment groups across all measured parameters.

**Table 6**. Ratios of seropositive/total number of pigs sampled per group at different time-points as tested with the IDEIA, *M. hyopneumoniae* EIA kit (Oxoid) are presented together with the inhibition percentages (IP). The contribution of each fattening unit (F1 and F2) on the combined (both units) seropositivity ratios and IP at 18 and 26 weeks of age is presented separately.

| | **Seropositive pigs for *M. hyopneumoniae* /total number of pigs sampled** | | | | **Average IP values** | | | |
|---|---|---|---|---|---|---|---|---|
| Age (w) | V1 | V2 | NV | P value | V1 | V2 | NV | P value |
| 2 | 7/20 | 10/20 | 9/20 | 0.624 | 44.2 | 42.6 | 45.6 | 0.929 |
| 10 | 3/20 | 0/20 | 0/20 | 1.000 | $49.9^{A}$ | $43.4^{AB}$ | $35.9^{B}$ | 0.004 |
| 18 (both units) | 5/20 | 4/20 | 3/20 | 0.735 | 39.0 | 47.4 | 29.3 | 0.066 |
| 26 (both units) | 18/20 | 16/20 | 16/20 | 0.630 | 82.6 | 72.3 | 68.1 | 0.159 |
| (F1) | | | | | | | | |
| 18 | 2/10 | 2/10 | 0/10 | 0.980 | $33.4^{AB}$ | $39.1^{B}$ | $14.3^{A}$ | 0.011 |
| 26 | 9/10 | 9/10 | 7/10 | 0.246 | $84.3^{A}$ | $77.1^{B}$ | $52.4^{B}$ | 0.006 |
| (F2) | | | | | | | | |
| 18 | 3/10 | 2/10 | 3/10 | 0.863 | 44.6 | 55.7 | 44.2 | 0.682 |
| 26 | 9/10 | 7/10 | 9/10 | 0.421 | 81.0 | 67.6 | 83.8 | 0.358 |

Treatment groups: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (non-vaccinated group). Within a row, different superscript letters correspond to significant differences in the average IP values between groups during the different sampling points ($P<0.05$).

**Table 7**. Ratios of qPCR positive animals for *Mycoplasma hyopneumoniae* (*Mhyo*)/total number of pigs sampled per group at different time-points are presented together with the Log *Mhyo* copies/mL. The contribution of each fattening unit (F1 and F2) on the combined (both units) qPCR positivity ratios and Log *Mhyo* copies/mL at 14 and 18 weeks of age is presented separately.

| | **qPCR positive pigs for *M. hyopneumoniae* /total number of pigs sampled** | | | | **Log *Mhyo* copies/mL** | | | |
|---|---|---|---|---|---|---|---|---|
| Age (w) | V1 | V2 | NV | P value | V1 | V2 | NV | P value |
| 10 | $0/20^{A}$ | $3/20^{B}$ | $17/20^{BC}$ | 0.000 | $0.58\pm0.71^{A}$ | $0.96\pm0.75^{AB}$ | $1.12\pm0.77^{C}$ | 0.000 |
| 14 (both units) | $2/20^{A}$ | $7/20^{B}$ | $10/20^{BC}$ | 0.041 | $0.85\pm0.72^{A}$ | $1.21\pm1.20^{BC}$ | $1.76\pm1.59^{C}$ | 0.000 |
| 18 (both units) | 2/20 | 8/20 | 8/20 | 0.088 | $1.99\pm2.45^{AB}$ | $2.21\pm2.44^{AC}$ | $2.19\pm2.35^{A}$ | 0.039 |
| (F1) | | | | | | | | |
| 14 | 2/10 | 2/10 | 7/10 | 0.073 | 0.91±0.25 | 1.05±1.21 | 1.62±1.38 | 0.257 |
| 18 | 1/10 | 6/10 | 4/10 | 0.183 | 2.03±2.50 | 1.78±2.03 | 2.28±2.32 | 0.076 |
| (F2) | | | | | | | | |
| 14 | 0/10 | 5/10 | 3/10 | 0.613 | $0.78\pm1.18^{A}$ | $1.33\pm1.17^{AB}$ | $1.86\pm1.69^{B}$ | 0.001 |
| 18 | 1/10 | 2/10 | 4/10 | 0.205 | 1.96±2.42 | 2.42±2.84 | 2.07±2.37 | 0.118 |

Treatment groups: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (non-vaccinated group). Within a row, different superscript letters indicate significant differences in the percentage of qPCR positive animals and qPCR values between groups during the different sampling points ($P<0.05$).

**Discussion**

The present field study investigated the efficacy of one-dose vaccination against *M. hyopneumoniae*, applied either three days before weaning (V1) or at the day of weaning (V2). The working hypothesis was that group V1 will perform better than group V2 across the different parameters investigated as the possible negative effect of weaning stress on the immunological responses developing after vaccination would be eliminated. Differences between group V1 and groups V2 and NV (non-vaccinated) were mostly not statistically significant, except for the second fattening unit (F2) where group V1 had significantly higher weight gains and ADG compared to groups V2 and NV between 10 and 27 weeks of age, as well as the whole study period. When taking into account both fattening units, group V1 had a numerically lower percentage of pigs with pneumonia, fissures and pleurisy at slaughter compared to groups V2 and NV. The results of this study are in agreement with the previous experimental study of Arsenakis *et al*. (2016; **section 3.1.**) where group V1 performed better than V2 across all parameters concerning the assessment of lung lesions, however differences between V1 and V2 were small and mostly statistically not significant.

In the present field study, serology and qPCR were both used to confirm the presence of pigs naturally infected with *M. hyopneumoniae*. At 26 weeks of age (1 week before slaughter) the percentage of tested pigs being seropositive for *M. hyopneumoniae* ranged between 70 and 90%, while at 18 weeks of age, between 10 and 60% of the tested pigs were found to be qPCR positive. These results indicate that *M. hyopneumoniae* has been involved as an important respiratory pathogen in both fattening units. Martínez *et al*. (2009) studied the relationship between infectious factors and pneumonia at slaughter on 39 fattening herds, and confirmed that vaccination of piglets against *M. hyopneumoniae* did not appear to be related with the seroprevalences against the pathogen at slaughter. The qPCR results indicate that infection with *M. hyopneumoniae* started already during the nursery period, which may explain the high prevalence of pulmonary fissures at

slaughter. Nevertheless, the diagnostic results show that apart from *M. hyopneumoniae*, there was a combination of viruses and other bacteria circulating in this herd.

In this study, it was decided to use the scoring system of Morrison *et al*., (1985a), in order to quantify the severity of macroscopically visible *Mycoplasma*-like lung lesions. Those lesions are typically compatible with catarrhal bronchopneumonia (CBPn), which is the most common lung lesion associated with *M. hyopneumoniae* infection (Sørensen *et al*., 2006; Meyns *et al*., 2011). It is characterized by well demarcated red-purplish areas that have a poor retraction state and thus, this scoring system was considered to be able to achieve the maximum possible differentiation against other types of lung lesions that are usually induced by viral pathogens, namely PRRSV, SIV and PCV-2. Those pathogens most often cause interstitial pneumonia (IPn; Van Alstine, 2012). The main difference between CBPn and IPn is that in the latter one the lesions are widely distributed throughout the lungs and the lung lobes maintain their rubbery consistency (Van Alstine, 2012; López and Martinson, 2017). It is documented that *M. hyopneumoniae* predisposes to secondary pathogens such as *T. pyogenes*, *P. multocida* and *S. suis* (Goodwin *et al*., 1965; Morisson *et al*., 1985b; Ciprian *et al*., 1988; Amass *et al*., 1994; Opriessnig *et al*., 2011; López and Martinson, 2017). By that way, the lung lesions produced by *M. hyopneumoniae* are exacerbated, leading to more severe CBP and pleural adhesions which extent the healing period (formation of fissures). Additionally, *M. hyopneumoniae* together with the aforementioned pathogens form the pathological complex of PEP (Maes *et al*., 1996; Thacker and Minion, 2012). Vaccination against *M. hyopneumoniae* in herds facing severe PEP has been shown to reduce the extent and severity of *Mycoplasma*-like lung lesions (Maes *et al*., 1999; Alexopoulos *et al*., 2004; Del Pozo Sacristán *et al*., 2013).

In the current study the percentage of pigs being positive for *M. hyopneumoniae* by qPCR increased between 14 and 18 weeks of age and the percentage of pigs seropositive for *M. hyopneumoniae* increased between 18 and 26 weeks of age. Additionally, in both F1 and F2, the

RDS increased between 20 and 26 weeks of age when compared to the period between 10 and 20 weeks of age. This increase in the RDS occurred almost simultaneously with the increase in the percentage of *M. hyopneumoniae* seropositive animals towards the end of the fattening period. Nathues *et al*. (2012), who examined the value of clinical examination in diagnosing PEP in fattening pigs from 59 herds at 18 weeks of age, suggested that a combination of an increasing RDS towards the end of the fattening period together with a seroprevalence of more than 50% against *M. hyopneumoniae* is highly indicative of an PEP diagnosis. In the current study, the PEP diagnosis is also justified by the detection of secondary pathogens, such as *T. pyogenes*, *P. multocida* and *S. suis*, in the pigs that were necropsied during the fattening period. The average percentage of *Mycoplasma*-like pneumonia lesions, in both F1 and F2, was considerably higher (38%) than the average of 24% reported by Meyns *et al*. (2011), who collected data from 50 randomly selected batches from 60 Belgian herds at slaughter.

Of course, it is important to mention that viral pathogens, such as PRRSV, SIV and PCV-2, were present in both fattening units (as documented by the diagnostics performed). The presence of such coinfections confirms the multifactorial nature of PRDC in the present herd and could probably explain why in most of the measured parameters the vaccinated groups V1 and V2 did not perform significantly better than the NV group. The high percentage of *Mycoplasma*-like pneumonia lesions among all groups can likely be the result of interactions between *M. hyopneumoniae* and the viral (PRRSV, SIV and PCV-2) pathogens observed. All tested pigs were seropositive for PRRSV and PCV-2 IgG at 20, 23 and 26 weeks of age, and additionally a high number of pigs were seropositive for PCV-2 IgM at 26 weeks of age. Given that all pigs participating in this trial did not receive any other vaccination apart from the one with the commercial *M. hyopneumoniae* bacterin, these results prove that the herd was facing a chronic PRRSV infection in combination with a circulating PCV-2 infection at the nursery and fattening units.

It is generally acknowledged that most PRRSV and PCV-2 infections occurring at the end of the fattening period are subclinical and thus, this is the reason why in many cases no gross IPn lesions are detected at slaughter (Segalés *et al*., 2012; Pileri and Mateu, 2016). Nevertheless, the potentiating effect of the combination of viral pathogens with a *M. hyopneumoniae* infection on the severity of *Mycoplasma*-like lung lesions induced has already been experimentally described by Thacker *et al*. (1999; 2001), Opriessnig *et al*. (2004) and Dorr *et al*. (2007), and demonstrated under field conditions by Del Pozo Sacristán *et al*. (2013). It is possible that in this study a better control of viral pathogens such as the PRRSV, PCV-2 and SIV through better biosecurity and vaccination at an early age, would allow vaccination against *M. hyopneumoniae* to demonstrate significant benefits versus the non-vaccinated animals in terms of the severity of *Mycoplasma*-like lung lesions induced. Previously published studies performed in herds facing mixed infections with the above mentioned pathogens, showed that vaccination against PCV-2 (Raith *et al*., 2015; Duivon *et al*., 2016) and PRRSV (Revilla *et al*., 2006), in combination with vaccination against *M. hyopneumoniae,* further reduced the prevalence of *Mycoplasma*-like lung lesions at slaughter (Raith *et al*., 2015; Duivon *et al*., 2016), reduced mortality rates (Revilla *et al*., 2006) and increased the ADG during the fattening period (Duivon *et al*., 2016), when compared to *M. hyopneumoniae* vaccination alone.

At this point it should be noted that apart from the case of vaccinating against PRRSV with a modified live vaccine, there are no studies mentioning that vaccination against *M. hyopneumoniae* prior to weaning can interfere with the efficacy of vaccinations against other pathogens that are also performed during that period. In the case of PRRSV vaccination, the results from the experimental studies of Thacker *et al*. (2000) and Drexler *et al*. (2010) imply that upon homologous challenge with a US- or an EU- type of PRRSV strain, respectively, interference with *M. hyopneumoniae* vaccination can occur when the modified live PRRSV vaccine is based on a US-type PRRSV strain and not on an EU-type PRRSV strain. An explanation was that antigenic

differences between the two types of PRRSV strains inflict different immune reactions by the host after vaccination, thus creating a different degree of interference with *M. hyopneumoniae* vaccination.

The evolution of respiratory distress in F1 is more typical of a SIV outbreak as a clear increase in the RDS was observed at 20 weeks of age and lasted for almost a week. A similar increase in coughing severity during a SIV outbreak has already been described by Berckmans *et al*. (2015) after using microphones for monitoring respiratory distress in a Dutch herd over a three-month period. For the remaining fattening period up to 26 weeks of age, a significantly higher overall average RDS was observed in F1 compared to F2. Thacker *et al*. (2001) reported that concurrent infection with *M. hyopneumoniae* and SIV increased the severity and duration of PEP. Van Reeth *et al*. (1996) found that the clinical effects of PRRSV were exacerbated with concurrent infection with SIV. Also, it is well established that secondary infections with bacteria such as *P. multocida* and *S. suis* may enhance the severity of a SIV outbreak (Van Reeth *et al*., 2012). Considering all the above and also the fact that there are many between-pathogen interactions which have not yet been fully elucidated, the higher RDS observed in F1 compared to F2 as well as the inconsistency in the results of group V1 for some of the measured parameters (such as the weight gains and ADG across the different fattening units) could possibly be attributed to the SIV outbreak. The proximity of F1 to the sow herd and the nursery unit provides a possible explanation for the origin of the SIV outbreak.

At 10 and 14 weeks of age, group V1 had significantly less animals being qPCR positive for *M. hyopneumoniae* than groups V2 and NV. Nevertheless, the impact of this lower colonization rate on the performance and clinical parameters remains unclear. A limitation of the current study is that this lower colonization rate together with the significantly lower average qPCR values (Log *M. hyopneumoniae* copies/mL) detected in group V1 comparing to groups V2 and NV could neither be confirmed prior to slaughter, since there were no TBS obtained at 26 or 27 weeks of

age. In a field study of Sibila *et al*. (2007), which compared two different *M. hyopneumoniae* vaccination programs (two vaccine doses at 1 and 3 weeks of age versus one dose at 6 weeks of age) to a control group, vaccination was related with a reduction in the number of animals found to be nested PCR positive at 25 weeks of age (37.5% and 55.8% of the vaccinated animals, respectively, and 70.2% of the control group). However, no quantitative method such as qPCR was used. It was concluded that a qPCR would be more useful in elucidating whether vaccination reduces the bacterial load in the upper respiratory airways during the late stages of the fattening period. Possibly the reduced bacterial load could be associated with the shedding of less organisms. This could be interesting to investigate in a future experimental trial, where vaccinated and non-vaccinated fattening pigs would be purchased from a farrow-to-finish herd and then involved in a transmission experiment using direct nose-to-nose contact.

Although this study included a high number of animals, results should be interpreted with caution, particularly when trying to draw general conclusions from a single study. It should always be taken into account that biosecurity levels, climatic environment and management conditions as well as metaphylactic antimicrobial treatment and vaccination programs differ between herds facing concurrent infections with *M. hyopneumoniae* and other respiratory pathogens. The above mentioned parameters influence not only mixed respiratory tract infection dynamics (Bochev, 2007; 2008; Thacker and Minion, 2012), but also the efficacy of the vaccination protocols applied against *M. hyopneumoniae* (Maes *et al*., 2008). Therefore, more herds with mixed respiratory disease where *M. hyopneumoniae* is involved as an important pathogen need to be investigated, in order to discover the most suitable techniques for making vaccination protocols that are tailored to the individual needs of each herd.

In conclusion, the fact that in F2 group V1 had significantly higher weight gains and ADG compared to groups V2 and NV is indicative of the potential benefits of vaccinating against *M. hyopneumoniae* three days before weaning. The SIV outbreak in F1 and the presence of other

respiratory pathogens in both F1 and F2 likely influenced the performance of both vaccinated groups and highlight the difficulties of evaluating interventions in field settings. Thus, additional studies are necessary to further explore the possible impact of the process of weaning on the efficacy of vaccination against *M. hyopneumoniae*.

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## 3.3. Effects of pre-farrowing sow vaccination against *Mycoplasma hyopneumoniae* on offspring colonization and lung lesions

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**Abstract**

This study investigated *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) colonization and lung lesions at slaughter in pigs from vaccinated (V) and non-vaccinated (NV) sows, in two herds (A and B). In each herd, two sow batches were V against *M. hyopneumoniae* with a commercial bacterin at six and three weeks before farrowing and two sow batches remained NV. From each sow batch, laryngeal swabs were collected from the litters of five primiparous sows at weaning and seven days post-weaning. All samples were tested for *M. hyopneumoniae* by nested PCR. In total, 488 piglets were sampled. Upon slaughter, the extent of *Mycoplasma*-like lung lesions (lung lesion score; LLS) was assessed. The colonization rates with *M. hyopneumoniae* at weaning and seven days post-weaning were (V-A=14.2, NV-A=20.0, (P=0.225); V-B=0.9, NV-B=0.8, (P=0.948)) and (V-A=0.8, NV-A=7.0, (P=0.039); V-B=1.8, NV-B=2.5, (P=0.738)), respectively. The average LLS (in %) was V-A=15.5, NV-A= 26.4, (P=0.021); V-B=9.7, NV-B=8.4, (P=0.541). In conclusion, in herd A, with a substantially higher level of piglet colonization at weaning than herd B, offspring from V sows had a significantly lower colonization rate seven days post-weaning and a significantly lower LLS at slaughter when compared to the offspring of the NV sows. This implies that sow vaccination might be useful for control of *M. hyopneumoniae* infections.

**Keywords**: *Mycoplasma hyopneumoniae*; Sow vaccination; Pig colonization; Lung lesions.

## Introduction

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the causative agent of porcine enzootic pneumonia and one of the most prevalent and important agents associated with the porcine respiratory disease complex (PRDC; Thacker and Minion, 2012). Infections with *M. hyopneumoniae* occur in almost all swine-producing areas around the world, causing major economic losses to the swine industry (Maes *et al*., 2008). Vaccination is one of the ways to control *M. hyopneumoniae* infections in pigs (Maes *et al*., 2008). The most common vaccination strategy in practice is vaccination of the piglets during suckling or at weaning. Single vaccination or double vaccination strategies are used, and in general vaccination results in significant improvements of clinical signs and lung lesions, lower performance losses and less antimicrobial use for treating *M. hyopneumoniae* infections (Del Pozo Sacristán *et al*., 2014a).

Concerning the breeding sow population, in some herds the gilts are vaccinated against *M. hyopneumoniae* during the quarantine period upon purchase, prior to moving them to the sow breeding facilities (Roos *et al*., 2016). This practice is part of the gilt acclimatization protocols applied in *M. hyopneumoniae* positive herds. It aims to avoid the destabilization of the breeding stock immunity by decreasing the bacterial load and the clinical signs in the vaccinated gilts (Maes *et al*., 2017; Takeuti *et al*., 2017). Vaccination of gestating sows against *M. hyopneumoniae* is not frequently practiced under field conditions (Sibila *et al*., 2008). Nevertheless, breeding sows are responsible for maintaining *M. hyopneumoniae* infections within the herds (Calsamiglia and Pijoan, 2000) and the percentage of piglets colonized with *M. hyopneumoniae* at weaning may be indicative of the number of sows shedding the pathogen during the suckling period (Ruiz *et al*., 2002). Additionally, it has been shown that low parity sows are more likely to transmit the pathogen to their piglets (Goodwin *et al*., 1965; Fano *et al*., 2006) and that they shed more *M. hyopneumoniae* organisms (Fano *et al*., 2006; Sibila *et al*., 2009), when compared to older sows. Finally, some studies suggest that in some herds the level of piglet colonization with *M.*

*hyopneumoniae* at weaning could be a predictor of the extent of *Mycoplasma*-like lung lesions at slaughter (Sibila *et al.*, 2008; Fano *et al*., 2007).

Thus, it is interesting to investigate whether vaccinating sows during gestation could decrease the percentage of their offspring that is found to be colonized with *M. hyopneumoniae* at weaning. In addition, it is not known whether this possible beneficial effect can still be observed at later time points, for example in the nursery units when piglets from different sows are mixed together, or even at slaughter age.

Therefore, a study was conducted in two Belgian herds having piglets colonized with *M. hyopneumoniae* at weaning. The objective was to investigate the effect of vaccinating gestating sows on the detection rates of *M. hyopneumoniae* at weaning and seven days after weaning as well as on the prevalence and extent of *Mycoplasma*-like lung lesions at slaughter.

## Materials and methods

### Herd description

The study was conducted between November 2015 and August 2016 in two farrow-to-finish herds: one multi-site (herd A) and one single-site (herd B). Herd A was managed by all-in/all-out (AIAO) across all production periods and operated a 4-week batch production system, while herd B only applied AIAO in the farrowing and the nursery units, and operated a 2-week batch production system. The pigs of herd A were weaned at 21 days of age, while those of herd B were weaned at 28 days of age. A description of both herds together with the health management practices applied are presented in Table 1.

Both herds were included in the study on the basis of a 10.0% minimum prevalence of colonization with *M. hyopneumoniae* at weaning. This cut-off point was chosen in order to match as much as possible with the prevalence of early piglet colonization reported in other studies (Calsamiglia and Pijoan, 2000; Ruiz *et al*., 2003; Villarreal *et al*., 2010). Prior to the onset of the study, a 10.0% prevalence rate was detected in each of the herds A and B by testing laryngeal swabs from 50 randomly selected pigs per herd. The pigs were sampled upon transfer to the nursery units and the swabs were tested for *M. hyopneumoniae* by nested PCR (n-PCR; Stärk *et al*., 1998). The number of 50 piglets sampled per herd allowed to detect with 95% certainty and 80% statistical power a mean prevalence of 10% *M. hyopneumoniae* positive piglets during the nursery period, if these herds faced early *M. hyopneumoniae* circulation.

**Table 1**. Herd description and health management practices.

| | **Herd A** | **Herd B** |
|---|---|---|
| Number of sows | 400 | 600 |
| Breed of sows | Topigs 20 | DanBred |
| Breed of boars (sperm) | Belgian Piétrain | Belgian Piétrain |
| **Vaccination of the sows** | | |
| Atrophic rhinitis | Rhiniseng (Hipra) 4weeks before farrowing | Porcilis AR-T (MSD) 4 weeks before farrowing |
| Parvovirus+*Erysipelothrix rhusiopathiae* | Eryseng Parvo (Hipra) 3 weeks before breeding | Porcilis Ery-Parvo (MSD) 2 weeks before breeding |
| *Escherichia coli* | Suiseng (Hipra) 3 weeks before farrowing | Porcilis coli (MSD) 3 weeks before farrowing |
| PRRS(V) | Ingelvac MLV (Boehringer) 8 weeks before farrowing | Ingelvac MLV (Boehringer) applied twice/year |
| **Vaccination of the quarantined gilts** | | |
| PRRS(V) | Ingelvac MLV (Boehringer) 4 weeks after arrival | Ingelvac MLV (Boehringer) 2 weeks after arrival |
| PCV-2 | Ingelvac Circoflex (Boehringer) twice 3 weeks apart | Ingelvac Circoflex (Boehringer) twice 3 weeks apart |
| Atrophic rhinitis | Rhiniseng (Hipra) twice 4 weeks apart | Porcilis AR-T (MSD) twice 6 weeks apart |
| **Management of the piglets** | | |
| (Days 2-4) | Amoxicillin (IM) | Amoxicillin (IM) |
| | Tail docking | Tail docking |
| | Castration | Castration |
| | Iron (Uniferon, Pharmacosmos) | Iron (Ferraject, Eurovet) |
| | Toltrazuril (Baycox, Bayer) | |
| Vaccinations | At weaning | N/A |
| PRRS(V) | Ingelvac MLV (Boehringer) | N/A |
| Mhyo and PCV-2 | Ingelvac FLEXcombo (Boehringer) | N/A |
| **Nursery** (day 21-70) | | |
| Medication | Amoxicillin days 21-28 (IF) | Zinc oxide after weaning for 14 days at 2500 ppm (IF) |
| Facilities | one unit with 4 compartments | one unit with 12 compartments |
| | 12 pens/compartment | 4 to 12 pens/compartment |
| | 40 pigs/pen (0.26 $m^2$/pig) | 20 to 26 pigs/pen (0.24 to 0.27 $m^2$/pig) |
| Ventilation | conventional mechanical ventilation | conventional mechanical ventilation |
| **Fattening unit** (day 70-220) | | |
| Medication | Flubendazole (day 70-75; IF), repeat every 6 weeks | Flubendazole (day 84 to 91 and day 110 to 117; IF) |
| Facilities | three fattening units, each having 4 compartments | two fattening units, each having 5 compartments |
| | 14 or 16 pens/compartment | 8 or 10 pens/compartment |
| | 12 or 15 pigs/pen (0.75 $m^2$/pig) | 10 or 15 pigs/pen (0.75 $m^2$/pig) |
| Ventilation | mechanical door ventilation | natural ventilation |

PRRSV, porcine reproductive and respiratory syndrome virus; PCV-2, porcine circovirus type 2; IM, intramuscular; N/A, non-applicable; IF, in-feed medication

**Study population and experimental design**

From four consecutive farrowing batches of sows in each herd, two batches were vaccinated (V) against *M. hyopneumoniae* and two remained non-vaccinated (NV). Vaccination was applied in an alternating way, so that one NV batch was followed by a V batch. In the V batches, all gilts and sows were intramuscularly injected on the lateral side of the neck, behind the ear, with a commercial one-shot bacterin (1 mL; Ingelvac MycoFLEX, Boehringer Ingelheim) at six and three weeks before the expected farrowing date. In each herd, the different farrowing batches participating in the study were named as follows: batch a (first batch, NV), batch b (second batch, V), batch c (third batch, NV) and batch d (fourth batch, V). Upon farrowing, from each NV or V batch of sows within each herd, five primiparous sows together with their litters were randomly selected. Primiparous sows were selected in order to increase the likelihood of finding piglets colonized with *M. hyopneumoniae* at weaning. Those primiparous sows had at least 10 piglets, and no addition of piglets from other sows to their litters was allowed. Removal of excessive piglets from those primiparous sows to other sows was allowed if considered necessary, but at least 10 piglets remained with their mother. All piglet removals were applied within the first two to three days after farrowing.

All piglets originating from the selected primiparous sows were ear-tagged at weaning, so that they could be identified throughout the whole production period until slaughter. In both herds, pigs housed within the same pen belonged to the same farrowing batch. At weaning, when moving pigs to the nursery units, they were partly regrouped according to pen size, weight and sex, while upon transfer to the fattening units, the pigs were regrouped according to pen size and weight. Within each nursery and fattening unit, the ear-tagged pigs were housed in different pens along with the rest of the pigs from the same farrowing batch. In Herds A and B, the pigs of the different production batches participating in the study were distributed to three and two different fattening units, respectively (Table 2). In both herds, all antimicrobial treatments applied during the study

were β-lactam antibiotics. These antibiotics are not active against *M. hyopneumoniae* as the bacterium lacks a cell wall. The study was approved by the ethical committee for animal experiments of the Faculty of Veterinary Medicine, Ghent University (EC2014/156).

**Table 2.** Distribution of the fattening pigs that originated from the different farrowing batches (a, b, c and d) in each fattening unit per herd.

| | **Herd A** | **Herd B** |
|---|---|---|
| **Batch** | **Fattening unit** | **Fattening unit** |
| a | 1 | 1 |
| b | 2 | 2 |
| c | 1 | N/A[1] |
| d | 3 | N/A[1] |

[1]Non-applicable as the pigs of batches c and d were not included in the statistical analysis for the lung lesions. The reason was that they were were slaughtered before the pre-arranged time and thus, no lung lesion evaluation was performed.

**Sample and data collection**

All samplings were conducted by the first author. Laryngeal swabs were collected from the selected primiparous sows and their piglets by the introduction of sterile swabs into the mouth cavity until they reached the larynx, using a mouth gag and a laryngoscope. The swabs used for sows were manufactured by Kruuse (Equivet uterine culture swab, product id: 290955), whereas a different type of swabs was used for their piglets (155C rayon, Copan Italia SpA). Sows were sampled 24 hours after the farrowing was completed. Piglets were sampled at weaning (prior to moving them in the nursery units) and seven days after weaning. In herd A, the piglets were vaccinated against *M. hyopneumoniae* on the day of weaning, after the samplings had been completed.

Oral fluid samples were collected from each pen hosting pigs originating from the selected sows at seven days after weaning. Sampling was done with cotton ropes (Swine oral fluids kit, Tego), using a ratio of one rope for each 25 pigs with a 30 min exposure (Hernandez-Garcia *et al*., 2017). The bottom part of each rope was adjusted so as to match with the average height of the pigs' shoulder joint. Oral fluid samples were collected into individual containers.

Blood samples were collected from the selected primiparous sows and their pigs at 24 hours after farrowing and at weaning, respectively. For the pigs originating from the selected litters in each farrowing batch, the extent (total area) of macroscopically visible *Mycoplasma*-like lung lesions (lung lesion score; LLS), and the prevalence of *Mycoplasma*-like pneumonia lesions, fissures and pleurisy were recorded at slaughter.

**Sample processing and testing**

Upon collection, all laryngeal swabs were immediately cooled at 4.0°C and subsequently stored at -20.0°C until analysis. DNA was extracted using a DNeasy Kit manufactured by Qiagen (Blood and Tissue kit, Belgium) according to the protocol used for buccal swabs. Detection of *M. hyopneumoniae* DNA by n-PCR was performed according to Stärk *et al*. (1998). The n-PCR products were analyzed by gel electrophoresis on a 1.5% agarose gel in Tris-Borate-EDTA (TBE) buffer and stained with GelRed (Biotium Inc.) with visualization under UV illumination. Extracted DNA from a pure culture of the virulent *M. hyopneumoniae* F7.2C field strain was used as a positive control (Arsenakis *et al*., 2016; **section 3.1.**).

During sampling, the individual containers with the oral fluid samples were cooled at 4.0°C and stored at -20.0°C until analysis. Oral fluid samples from each pen were pooled after thawing and let to stand for one hour at room temperature. Then, the samples were centrifuged for 10 minutes at 80g and the supernatant was collected. Following the aforementioned centrifugation step, all supernatants were centrifuged for 30 minutes at 13500g. The collected pellets were then used to

perform the DNA extractions using the DNeasy Kit manufactured by Qiagen (Blood and Tissue kit, Belgium) according to the protocol used for blood or body fluids. Detection of *M. hyopneumoniae* DNA by n-PCR was performed as described for the laryngeal swabs.

All blood samples collected were used to measure antibodies against *M. hyopneumoniae via* a blocking ELISA (IDEIA, *M hyopneumoniae* EIA Kit, Oxoid, UK) as previously described by Del Pozo Sacristán *et al*. (2014b). This ELISA is based on monoclonal antibodies against a 74 kDa protein of *M. hyopneumoniae* (Feld *et al*., 1992). Inhibition percentages (IP) for all sera were calculated considering the optical density value of each serum sample as well as the negative control according to Sibila *et al*. (2004). Classification of individual sera on the basis of IP values was as follows: IP <30.0%, negative; IP >50.0%, positive; IP ≥30.0% and ≤50.0%, equivocal.

At slaughter, the LLS was quantified according to the scoring system described by Morrison *et al*. (1985) and Del Pozo Sacristán *et al*. (2012; 2014b). *Mycoplasma*-like pneumonia lesions (catarrhal bronchopneumonia (CBPn)) were defined as red to purplish areas of cranioventral consolidation raised above the surface or at the surface of each lobe and with a liver-like consistency. Chronic *Mycoplasma*-like pneumonia lesions (fissures) were grey to purplish cranioventral scars, shrunken below the surface of the lobes. Pleurisy was evident as fibrous adhesions between the lung lobes and/or the lungs and thoracic wall.

**Statistical analysis**

With regard to the detection rates of *M. hyopneumoniae* in laryngeal swabs at weaning, the inclusion of a minimum of 55 piglets from the selected primiparous sows per farrowing batch allowed to detect with 95% certainty and 80% statistical power a difference of 10.5 percentage points between pigs of the NV and V sow batches. The inclusion of a minimum of 55 pigs in each farrowing batch allowed to assess a difference of 4.6 points in LLS (SD=12.50) with 95% certainty and 80% statistical power (IBM SPSS Sample Power 3, Illinois, USA). The primary

outcome parameters were the detection rates of *M. hyopneumoniae* in the swabs obtained from the selected sows at farrowing as well as the swabs taken from their piglets at weaning and seven days after weaning, and the LLS of those pigs at slaughter. The secondary parameters were the percentage of nursery pens where *M. hyopneumoniae* detection occurred seven days after weaning by oral fluids, and the percentage of sows and piglets showing *M. hyopneumoniae* antibodies at farrowing and weaning, respectively. *M. hyopneumoniae* detection rates in laryngeal swabs were analyzed using binary logistic regression with vaccination status (NV or V) and farrowing batch as predictors for the model. Logistic regression was also used to compare between individual farrowing batches the percentage of nursery pens that were positive for *M. hyopneumoniae* by oral fluids as well as the percentage of seropositive sows and piglets. In herd A, the percentage of pigs showing *Mycoplasma*-like pneumonia lesions, fissures and pleurisy between individual farrowing batches were also analyzed *via* logistic regression, including the fattening unit as a predictor for the model.

Fisher's exact test was used to analyze the percentage of nursery pens positive for *M. hyopneumoniae* by oral fluids according to the vaccination status of their piglets. A binary categorical variable was created to indicate the percentage of piglets originating from pens positive by oral fluids and subsequently, to correlate this percentage with the *M. hyopneumoniae* detection rates in laryngeal swabs obtained from the same piglets at seven days after weaning. Fisher's exact test was also used to analyze the total percentage of seropositive sows and piglets between the NV and V farrowing batches as well as the total percentage of pigs with *Mycoplasma*-like pneumonia lesions, fissures and pleurisy. *M. hyopneumoniae* detection rates in laryngeal swabs obtained from the piglets at weaning and seven days after weaning as well as the percentage of seropositive piglets at weaning were correlated *via* Spearman's rank correlation with the LLS of the same pigs at slaughter. Kruskal-Wallis was used to analyze data that did not fulfil the criteria of normality and homogeneity of variances, namely the serology IP and the LLS. For all the above

mentioned analyses, statistical results were considered significant when P-values were ≤ 0.05 (two-sided test). The statistical package SPSS V.23.0 was used to analyze the data.

## Results

Detection of *M. hyopneumoniae* using n-PCR in laryngeal swabs

In both herds, no statistically significant differences were found in the percentage of the selected n-PCR positive sows at farrowing between the NV and V batches (Herd A: P=0.999; Herd B: P=0.608, Table 3). The same was observed for the comparisons between individual sow batches (Herd A: P=0.999; Herd B: P=0.902, Table 3).

Concerning the percentage of n-PCR positive piglets at weaning, in both herds there were no significant differences between the piglets of the selected NV and V sows (Herd A: P=0.225; Herd B: P=0.948, Table 4). In herd A, significantly more piglets originating from the selected NV sows were found to be n-PCR positive at seven days after weaning when compared to those of the selected V sows (P=0.039, Table 4). In herd B, there were no significant differences between the selected NV and V sows in the percentage of n-PCR positive piglets at seven days after weaning (P=0.738, Table 4).

Detection of *M. hyopneumoniae* using n-PCR in oral fluids

The percentages of nursery pens that were positive for *M. hyopneumoniae* by oral fluids in herds A and B are presented in Table 5. In both herds, no significant differences between the pens hosting piglets of the selected NV and V sows were detected (Herd A: P=0.429; Herd B: P=1.000).

In herd A, a significantly higher percentage of piglets originating from pens that were *M. hyopneumoniae* positive by oral fluids were found to be positive by laryngeal swabs seven days after weaning when compared to the piglets originating from pens that were *M. hyopneumoniae* negative by oral fluids (P=0.001). No such differences were observed in herd B (P=0.998).

**Table 3**. Percentage and number (in parentheses) of sows with a *M. hyopneumoniae* nested-PCR positive laryngeal swab.

| | | **Herd A** | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | a | b | c | d | P value | a-c (NV) | b-d (V) | P* value |
| **Farrowing** | 0.0 (0/5) | 0.0 (0/5) | 20.0 (1/5) | 0.0 (0/5) | 0.999 | 10.0 (1/10) | 0.0 (0/10) | 0.999 |
| | | **Herd B** | | | | | | |
| | a | b | c | d | P value | a-c (NV) | b-d (V) | P* value |
| **Farrowing** | 0.0 (0/5) | 40.0 (2/5) | 40.0 (2/5) | 20.0 (1/5) | 0.902 | 20.0 (2/10) | 30.0 (3/10) | 0.608 |

Vaccination status: NV (non-vaccinated sow batches) and V (vaccinated sow batches). The P value refers to the comparisons between the individual sow batches and the P* value to the comparisons between the summaries of the NV (a-c) and V (b-d) batches. Differences between batches were not statistically significant (P>0.05).

**Table 4**. Percentage and number (in parentheses) of piglets with a *M. hyopneumoniae* nested-PCR positive laryngeal swab.

| | | | **Herd A** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Age (weeks) | a | b | c | d | P value | a-c (NV) | b-d (V) | P* value |
| **Weaning** | **3** | 25.4 (15/59) | 14.9 (10/67) | 14.7 (9/61) | 13.3 (8/60) | 0.277 | 20.0 (24/120) | 14.2 (18/127) | 0.225 |
| **Nursery period** | **4** | 12.5 (7/56) | 0.0 (0/66) | 1.7 (1/59) | 1.8 (1/57) | 0.088 | 7.0 (8/115)$^{A}$ | 0.8 (1/123)$^{B}$ | 0.039 |
| | | | **Herd B** | | | | | | |
| | Age (weeks) | a | b | c | d | P value | a-c (NV) | b-d (V) | P* value |
| **Weaning** | **4** | 0.0 (0/65) | 0.0 (0/57) | 1.6 (1/61) | 1.7 (1/58) | 0.997 | 0.8 (1/126) | 0.9 (1/115) | 0.948 |
| **Nursery period** | **5** | 3.1 (2/64) | 1.8 (1/55) | 1.7 (1/58) | 1.8 (1/55) | 0.943 | 2.5 (3/122) | 1.8 (2/110) | 0.738 |

Vaccination status: NV (pigs originating from non-vaccinated sow farrowing batches) and V (pigs originating from vaccinated sow farrowing batches). The P value refers to the comparisons between the individual farrowing batches and the P* value to the comparisons between the summaries of the NV (a-c) and V (b-d) batches. Values with different superscripts within a row are significantly different ($P<0.05$).

**Table 5**. Percentage and number (in parentheses) of pen-based oral fluid samples positive by nested-PCR for *M. hyopneumoniae*.

| | | | **Herd A** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Age (weeks) | a | b | c | d | P value | a-c (NV) | b-d (V) | P* value |
| **Nursery period** | **4** | 100.0 (2/2) | 0.0 (0/2) | 0.0 (0/2) | 0.0 (0/2) | N/A[1] | 50.0 (2/4) | 0.0 (0/4) | 0.429 |
| | | | **Herd B** | | | | | | |
| | Age (weeks) | a | b | c | d | P value | a-c (NV) | b-d (V) | P* value |
| **Nursery period** | **5** | 0.0 (0/9) | 0.0 (0/7) | 25.0 (2/8) | 12.5 (1/8) | 0.941 | 11.8 (2/17) | 6.7 (1/15) | 1.000 |

Vaccination status: NV (pens hosting pigs from non-vaccinated sow farrowing batches) and V (pens hosting pigs from vaccinated sow farrowing batches). The P value refers to the comparisons between the individual farrowing batches and the P* value to the comparisons between the summaries of the NV (a-c) and V (b-d) batches. Differences between batches were not statistically significant (P>0.05).

[1]Non-applicable due to insufficient number of observations.

Serological investigation

The serological results for *M. hyopneumoniae* are presented in Table 6. In both herds, the percentage of selected sows that were seropositive at farrowing was influenced by their vaccination status. The NV batches exhibited a significantly lower percentage of sows that were seropositive than the V batches (Herd A: P=0.001; Herd B: P=0.023). The same trend was observed regarding the differences in the IP values (Herd A: P=0.000; Herd B: P=0.017). At weaning, a significantly lower percentage of seropositive piglets originated from the selected NV sows when compared to the selected V sows (both Herds A and B: P=0.000). Similarly, the piglets of the selected NV sows exhibited lower average IP values than those of their V counterparts (both Herds A and B: P=0.000).

In both herds, there was no significant difference between the percentages of seropositive and seronegative piglets that were found to be n-PCR positive at weaning (Herd A: P=0.145; Herd B: P=1.000). In herd A, a significantly lower percentage of piglets being seropositive at weaning were found to be n-PCR positive at seven days after weaning when compared to their seronegative counterparts (P=0.024). In herd B, no such difference was observed (P=1.000).

Lung lesions

In herd A, lung lesion evaluation at slaughter was performed on 68/120 and 64/127 pigs that originated from the selected NV and V sows (across batches a, b, c and d), respectively (Table 7). Lost ear tags was the reason why not all pigs could be evaluated. In herd B, only batches a and b were investigated at slaughter, with the lung lesion evaluation performed on 47/65 and 55/57 pigs originating from the selected sows, respectively (Table 7). Pigs that originated from batches c and d were, due to miscommunication, already slaughtered before the pre-arranged time.

In both herds, logistic regression analyses showed that the different fattening units did not have a significant effect on the prevalence of *Mycoplasma*-like pneumonia lesions and fissures. In herd

A, significant differences between the different production batches were only obtained for the prevalence and extent of *Mycoplasma*-like pneumonia lesions. More specifically, a significantly higher percentage of pigs originating from the NV sows had *Mycoplasma*-like pneumonia lesions when compared to those of the V sows (P=0.045, Table 7). The pigs of the NV sows exhibited a significantly higher LLS than those of the V sows (P=0.021, Table 7). In herd B, significant differences between the pigs of the NV and V sows were only observed for the prevalence of pleurisy, which was higher in the pigs of the NV sows (P=0.000, Table 7).

In both herds, there was no significant difference in the prevalence of *Mycoplasma*-like pneumonia lesions at slaughter between the pigs that were n-PCR positive at weaning (Herd A: P=0.125; Herd B: P=0.238) or seven days after weaning (Herd A: P=0.680; Herd B: P=1.000) and their n-PCR negative counterparts. Concerning the LLS, there was no significant difference between the pigs that were n-PCR positive at weaning (Herd A: $r^2$=0.156, P=0.143; Herd B: $r^2$=-0.036, P=0.714) or seven days after weaning (Herd A: $r^2$=0.123, P=0.252; Herd B: $r^2$=-0.052, P=0.603) and their n-PCR negative counterparts.

In herd A, a significantly lower prevalence of *Mycoplasma*-like pneumonia lesions (P=0.048) and a lower LLS ($r^2$=-0.267, P=0.011) at slaughter was observed in the pigs being seropositive at weaning when compared to their seronegative counterparts. In herd B, no such difference was observed for the prevalence of *Mycoplasma*-like pneumonia lesions (P=0.484) and the LLS ($r^2$=0.025, P=0.804).

**Table 6**. Percentage and number (in parentheses) of seropositive sows and pigs at farrowing and weaning, respectively, together with the inhibition percentages (IP).

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Herd A** | | | | | | | | | |
| | Stage | a | b | c | d | P value | a-c (NV) | b-d (V) | P* value |
| **Sows seropositive** | **Farrowing** | 20.0 (1/5) | 80.0 (4/5) | 0.0 (0/5) | 100.0 (5/5) | 0.380 | 10.0 (1/10)$^{A}$ | 90.0 (9/10)$^{B}$ | 0.001 |
| **Sows IP** | **Farrowing** | 25.6$^{AB}$ | 77.5$^{ABC}$ | 26.8$^{B}$ | 94.4$^{C}$ | 0.005 | 26.2$^{A}$ | 86.0$^{B}$ | 0.000 |
| **Pigs seropositive** | **Weaning** | 6.8 (4/59)$^{A}$ | 74.6 (50/67)$^{B}$ | 5.1 (3/59)$^{C}$ | 90.0 (54/60)$^{D}$ | 0.000 | 5.9 (7/118)$^{A}$ | 81.9 (104/127)$^{B}$ | 0.000 |
| **Pigs IP** | **Weaning** | 15.6$^{A}$ | 68.1$^{B}$ | 29.0$^{A}$ | 79.6$^{B}$ | 0.000 | 22.3$^{A}$ | 73.2$^{B}$ | 0.000 |
| **Herd B** | | | | | | | | | |
| | Stage | a | b | c | d | P value | a-c (NV) | b-d (V) | P* value |
| **Sows seropositive** | **Farrowing** | 40.0 (2/5) | 80.0 (4/5) | 0.0 (0/4) | 80.0 (4/5) | 0.532 | 22.0 (2/9)$^{A}$ | 80.0 (8/10)$^{B}$ | 0.023 |
| **Sows IP** | **Farrowing** | 39.0$^{AB}$ | 56.4$^{AB}$ | 19.9$^{A}$ | 68.8$^{B}$ | 0.049 | 30.5$^{A}$ | 62.6$^{B}$ | 0.017 |
| **Pigs seropositive** | **Weaning** | 42.2 (27/64)$^{A}$ | 66.7 (38/57)$^{B}$ | 3.3 (2/60)$^{C}$ | 77.6 (45/58)$^{B}$ | 0.000 | 23.4 (29/124)$^{A}$ | 72.2 (83/115)$^{B}$ | 0.000 |
| **Pigs IP** | **Weaning** | 42.4$^{A}$ | 79.3$^{B}$ | 24.6$^{C}$ | 65.2$^{D}$ | 0.000 | 38.5$^{A}$ | 72.5$^{B}$ | 0.000 |

Vaccination status: NV (non-vaccinated sow batches and their pigs) and V (vaccinated sow batches and their pigs). The P value refers to the comparisons between the individual farrowing batches and the P* value to the comparisons between the summaries of the NV (a-c) and V (b-d) batches. Values with different superscripts within a row are significantly different ($P<0.05$ and $P^{*}<0.05$).

**Table 7**. Percentage of pigs with *Mycoplasma hyopneumoniae*-like lung lesions (*Mhyo*), fissures and pleurisy at slaughter, and lung lesion score (LLS; average±SD).

| | **Herd A** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | a (*n*=33) | b (*n*=33) | c (*n*=35) | d (*n*=31) | P value | a-c (NV; *n*=68) | b-d (V; *n*=64) | P* value |
| **Prevalence of *Mhyo*-like lesions** | $95.7^{A}$ | $65.2^{B}$ | $60.0^{B}$ | $47.6^{B}$ | 0.038 | $77.1^{A}$ | $56.8^{B}$ | 0.045 |
| **Prevalence of fissures** | 33.3 | 17.4 | 0.0 | 0.0 | 0.676 | 16.3 | 9.1 | 0.360 |
| **Prevalence of pleurisy** | 33.3 | 12.5 | 7.7 | 9.5 | 0.078 | 20.0 | 11.1 | 0.272 |
| **LLS** | $29.4±19.1^{A}$ | $14.9±16.7^{B}$ | $23.6±28.6^{AB}$ | $16.1±21.8^{B}$ | 0.032 | $26.4±24.4^{A}$ | $15.5±19.1^{B}$ | 0.021 |
| | **Herd B** | | | | | | | |
| | a (*n*=47) | b (*n*=55) | c | d | P value | a (NV; *n*=47) | b (V; *n*=55) | P* value |
| **Prevalence of *Mhyo*-like lesions** | 78.7 | 74.1 | $N/A^{1}$ | $N/A^{1}$ | $N/A^{1}$ | 78.7 | 74.1 | 0.649 |
| **Prevalence of fissures** | 46.8 | 37.9 | $N/A^{1}$ | $N/A^{1}$ | $N/A^{1}$ | 46.8 | 37.9 | 0.428 |
| **Prevalence of pleurisy** | 38.3 | 5.2 | $N/A^{1}$ | $N/A^{1}$ | $N/A^{1}$ | $38.3^{A}$ | $5.2^{B}$ | 0.000 |
| **LLS** | 8.4±8.5 | 9.7±11.6 | $N/A^{1}$ | $N/A^{1}$ | $N/A^{1}$ | 8.4±8.5 | 9.7±11.6 | 0.541 |

Vaccination status: NV (pigs originating from non-vaccinated sow farrowing batches) and V (pigs originating from vaccinated sow farrowing batches). The P value refers to the comparisons between the individual farrowing batches and the P* value to the comparisons between the summaries of the NV (a-c) and V (b-d) batches. Values with different superscripts within a row are significantly different ($P<0.05$ and $P*<0.05$).

[1]Non-applicable as pigs of batches c and d were slaughtered before the pre-arranged time and thus, no lung lesion evaluation was performed.

**Discussion**

The present study investigated whether *M. hyopneumoniae* vaccination of sows at the end of gestation influenced the *M. hyopneumoniae* colonization status of their piglets during the peri-weaning period as well as the prevalence and extent of *Mycoplasma*-like pneumonia lesions at slaughter. Results showed that in herd A, pigs originating from the selected V primiparous sows had a significantly lower percentage of colonized pigs at seven days post-weaning when compared to their counterparts from the selected NV primiparous sows. Additionally, at slaughter, the pigs of the selected V primiparous sows had a significantly lower prevalence of *Mycoplasma*-like pneumonia lesions and a significantly lower LLS than those of the selected NV primiparous sows. In herd B, there were no significant differences detected for any of the aforementioned parameters. This might be explained by the lower colonization pressure observed in herd B (Sibila *et al*., 2008).

Concerning the results obtained in herd A, it should be taken into account that double sow vaccination during late gestation was applied in addition to piglet vaccination at weaning. A different study design was used by Díaz *et al*. (2004), where the prevalence of *Mycoplasma*-like pneumonia lesions at slaughter was compared between one herd that vaccinated the sows in addition to piglet vaccination (herd 1) and one herd that vaccinated only the piglets (herd 2). At the beginning of the study, the prevalence of pneumonia lesions across herds 1 and 2 was 22 and 21%, respectively. After an eight-month implementation period, this prevalence was 7 and 18%, respectively. Nevertheless, this study did not include parameters such as the detection rates of *M. hyopneumoniae* in the piglets and the LLS at slaughter. Additionally, sows were vaccinated only once at 3 weeks before farrowing.

The studies of Ruiz *et al*. (2003) and Sibila *et al*. (2008) were conducted in herds that did not additionally vaccinate their piglets and utilized farrowing batches where half of the sows were NV

and half V. In both studies, the V sows received vaccination at five and three weeks before farrowing. The studies reported on the effect of sow vaccination on piglet colonization using a single sampling point either at weaning (Ruiz *et al*., 2003) or during the post-weaning period (Sibila *et al*., 2008), and additionally on the extent of *Mycoplasma*-like pneumonia lesions at slaughter (Sibila *et al*., 2008). It was shown that vaccination of the sows reduced the number of pigs colonized with *M. hyopneumoniae* (Ruiz *et al*., 2003; Sibila *et al*., 2008) and the extent of pneumonia lesions at slaughter (Sibila *et al*., 2008). Nevertheless, from the two consecutive production batches included in the study of Ruiz *et al*. (2003) significant differences in the colonization rates between the pigs originating from the NV and V sows were only obtained in the first batch, while in the study of Sibila *et al*. (2008) the differences in colonization across the single batch investigated were non-significant.

All the aforementioned differences between the present study and the studies of Ruiz *et al*. (2003), Díaz *et al*. (2004) and Sibila *et al*. (2008) show that still further investigations are needed in order to conclude which vaccination program (sow vaccination alone or in addition to piglet vaccination) is more efficient in reducing the number of piglets colonized with *M. hyopneumoniae* during the peri-weaning period and the lung lesions of these pigs at slaughter.

An additional difference of the present study with the aforementioned studies (Ruiz *et al*., 2003; Díaz *et al*., 2004; Sibila *et al*., 2008) is that only litters originating from primiparous sows were utilized, in order to increase the likelihood of finding piglets colonized with *M. hyopneumoniae* (Goodwin *et al*., 1965; Fano *et al*., 2006). Nevertheless, there was no correlation between the colonization of the sows and the colonization of their piglets. The predictability of the piglet colonization status based on the sow parity number alone may be misleading and still remains to be elucidated (Calsmamiglia and Pijoan, 2000; Sibila *et al*., 2007). In fact, *M. hyopneumoniae* is not only transmitted vertically from the sows to their piglets, but also horizontally between piglets sharing the same environment and belonging to different litters (during the suckling period) or

pens (during the nursery period) (Nathues *et al*., 2013). Thus, the results of the present study suggest that horizontal transmission might have played an important role in the colonization rates detected among the piglets of the primiparous sows.

In the current study it was decided to use laryngeal swabs as they were considered to be the most suitable method to conduct samplings in both sows and piglets (Pieters *et al*., 2017; Takeuti *et al*., 2017), over the whole duration of this longitudinal study that employed a higher number of animals. The colonization rates observed in the piglets of herd A decreased between weaning and seven days post-weaning. The reasons for this are not clear. Sibila *et al*. (2004) have observed a similar colonization pattern where the proportion of colonized piglets decreased with age after weaning. This scenario fits with previous suggestions that shedding of *M. hyopneumoniae* is an intermittent and of variable intensity process that still needs to be fully elucidated (Meyns *et al*., 2004; Sibila *et al*., 2009; Villarreal *et al*., 2011; Roos *et al*., 2016; Maes *et al*., 2017).

In both herds participating in this study, there were no significant differences in the percentage of nursery pens being positive for *M. hyopneumoniae* by oral fluids between the NV and the V production batches. In herd A, a significantly higher percentage of piglets from positive pens were found to be laryngeal swab-positive at seven days post-weaning when compared to the piglets originating from negative pens. In herd B, there was no such difference detected. Given that both types of samplings were conducted on the same day, those results mean that the consistency of detecting pens hosting colonized piglets by oral fluids was better in herd A than in herd B. This might be due to the fact that in herd A a higher number of positive piglets were distributed in less pens (nine positive piglets in six pens) when compared to herd B (five positive piglets in 32 pens). The results of this study agree with the studies of Hernandez-Garcia *et al*. (2017) and Pieters *et al*. (2017) that under field conditions *M. hyopneumoniae* can be inconsistently detected by oral fluids and thus, samplings from multiple pens and on multiple occasions may be needed.

In the present study, it was evident that an improved transfer of colostral antibodies was achieved in the piglets of the V sows when compared to their counterparts from the NV sows. In both herds, 0 to 40% of the NV sows were seropositive at farrowing *versus* 80 to 100% of the V sows. Those differences in the percentage of seropositive sows across the NV and V farrowing batches are in agreement with the studies of Ruiz *et al*. (2003) and Sibila *et al*. (2008). Additionally, in the present study a significantly lower percentage of seropositive piglets at weaning came from NV sows (ranging between 3 and 42%) when compared to the V sows (ranging between 67 and 90%). Those differences are also in agreement with previous studies of Ruiz *et al*. (2003) and Martelli *et al*. (2006) who used similar experimental designs and sampled piglets at the same age.

In herd A, a significantly higher number of piglets being seronegative at weaning were found to be n-PCR positive at seven days post-weaning when compared to their seropositive counterparts. Additionally, the seronegative piglets had a significantly higher prevalence and extent of *Mycoplasma*-like pneumonia lesions at slaughter than the seropositive ones. In herd B, no such differences were detected and an explanation might be that across all production batches investigated a low colonization pressure, together with low a LLS were observed. Pieters *et al*. (2014) found no relationship between piglet sero-status at weaning and colonization, nevertheless in that study all piglets originated from NV sows.

The aforementioned associations observed in herd A between the sero-status of the piglets at weaning and their n-PCR positivity at seven days post-weaning could be attributed to the lower passive transfer of maternal antibodies to the piglets of the NV sows when compared to their counterparts from the V sows. In fact, 8/9 piglets found to be n-PCR positive at seven days post-weaning were seronegative at weaning and originated from NV sows. No significant differences were obtained when the data from pigs originating from the NV and V sow batches were analyzed separately, likely because of reduced statistical power of the analyses. On a similar way in the same herd, this lower passive transfer of maternal antibodies to the piglets of the NV sows might

have played an important role in the differences observed between the seronegative and seropositive pigs in the development of *M. hyopneumoniae*-like pneumonia lesions. In fact, 51/89 pigs with *Mycoplasma*-like pneumonia lesions at slaughter were seronegative at weaning and originated from NV sows *versus* 22/89 that were seropositive at weaning and originated from V sows.

Colostral antibodies provide partial protection against both experimentally-induced and natural infections (Hodgins *et al*., 2004). In the present study, the passive transfer of maternal immunity was indirectly measured through an ELISA detecting the levels of serum IgG. Djordjevic *et al*. (1997) showed that IgG concentrations (in serum and respiratory tract washings) were not correlated with protection against *M. hyopneumoniae* infection. Thus, there must be additional immunological components, other than the IgG antibodies measured in the current and previous studies, present in the milk of the V sows, whose enhanced presence (due to vaccination) conferred improved resiliency to their pigs against colonization and *M. hyopneumoniae*-like pneumonia lesion development when compared to the pigs originating from the NV sows. These might include antibodies against *M. hyopneumoniae* antigens not measured in the ELISA used here, as well as immune cells. Indeed, Bandrick *et al*. (2008) conducted an experimental study and showed that lymphocytes that were passively transferred from V sows to their piglets *via* the colostrum were able to proliferate in *in vitro* recall assays and might participate in a functional response against *M. hyopneumoniae*.

Overall, in the present study vaccination of the primiparous sows in herd A (which had a sufficient number of colonized piglets at weaning when compared to herd B) reduced the number of colonized pigs at seven days post-weaning as well as the prevalence and extent of *Mycoplasma*-like pneumonia lesions at slaughter. Thus, sow vaccination could be a useful tool to control *M. hyopneumoniae* infections in herds that maintain a high proportion of primiparous sows and where colonized piglets are detected during the early production stages. Sow colonization at farrowing as

measured with the method used here, was not correlated with piglet colonization at weaning or post-weaning. In this context, the fact that the sero-status of the piglets had an effect on the colonization rates detected at seven days post-weaning, and the prevalence and extent of *Mycoplasma*-like pneumonia lesions at slaughter implies that immunological components passively transferred *via* the colostrum may play an important role. More studies are needed to elucidate the aforementioned immunological mechanisms of resiliency to colonization and infection as such is achieved by gestating-sow-vaccination.

## Competing interests

The study was financed by Boehringer Ingelheim but was conducted solely by the Unit Porcine Health Management at the Faculty of Veterinary Medicine of Ghent University. Gabriele Schagemann and Charles Oliver Gomez-Duran are employed by Boehringer Ingelheim Animal Health and were not involved in the collection, analysis and interpretation of the data.

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# General discussion

## GENERAL DISCUSSION

*Mycoplasma hyopneumoniae* continues to be one of the most prevalent respiratory pathogens in almost all countries with intensive pig production (Maes *et al*., 2017). Vaccination with commercial bacterins is one of the most common ways to control *M. hyopneumoniae* infections, together with the use of antimicrobials, and the optimization of the management practices and microclimate conditions (Thacker and Minion, 2012). Traditionally, the improvement of management practices and microclimate conditions are considered to be the first measure that should be taken in endemically infected herds and indeed, many herds have nowadays adopted all-in/all-out production sytems, optimal stocking densities and automated ventilation systems (Maes *et al*., 2008; Simionatto *et al*., 2013). Nevertheless, PEP-induced clinical disease is still prevalent in these herds and significant economic losses occur due to pig growth retardation, high feed conversion ratios and increased mortality rates due to the involvement of secondary pathogens (Martelli *et al*., 2006; Sibila *et al*., 2009).

To make it more complicated, often the application of an eradication program is not a feasable option to the herd owners and the veterinarians (Desrosiers, 2001; Holst *et al.*, 2015), for three main reasons: a) the risk of re-infection attributed to neighbouring infected herds or the purchase of infected replacement gilts is considerable, b) the requirement of depopulation and repopulation, which for large herds is often not a considerable option due to the duration of lost production, and/or c) the herd closure, which for one-site operations is limited in duration by the need to replace sow mortalities and culls, while for multi-site operations requires offsite breeding facilities. As a result, in many countries the majority of the herds rely on vaccination and antimicrobial medication, in order to achieve additional control of *M. hyopneumoniae* infections and further reduce the economic losses inflicted by PEP (Thacker and Minion, 2012; Maes *et al*., 2017). Nowadays, in several countries there are intensive efforts to reduce antimicrobial usage

(due to the occurrence of antimicrobial resistance in different bacterial pathogens), thus the optimization of the vaccination protocols against *M. hyopneumoniae* has gained significance.

The present thesis investigated different vaccination protocols in peri-weaned piglets and breeding sows, with the aim to reduce the shedding of the pathogen from the sows to the piglets as well as the severity of clinical disease and macroscopic *Mycoplasma*-like lung lesions in fattening pigs. The first study investigated whether the weaning process negatively influenced the efficacy of *M. hyopneumoniae* vaccination against experimental challenge infection (**section 3.1.**). The second study investigated whether the weaning process negatively influenced the efficacy of *M. hyopneumoniae* vaccination under field conditions, in a herd natually infected with *M. hyopneumoniae* and facing mixed respiratory tract infections by other pathogens (**section 3.2.**). Finally, a third study investigated whether breeding sow vaccination during late gestation could reduce the percentage of colonized offspring with *M. hyopneumoniae* at weaning and seven days after weaning, and whether this offspring exhibited reduced prevalence and severity of macroscopic *Mycoplasma*-like lung lesions at slaughter (**section 3.3.**).

This general discussion will focus on the findings obtained in the aforementioned studies of this thesis. It will successivley discuss: a) the results obtained for the major outcome parameters used to assess the efficacy of the vaccination protocols applied, and b) the role of the different sampling methods employed to demonstrate the presence of the pathogen in the different age groups. Finally, general conclusions and future research perpsectives will be provided.

***Effect of the weaning process on the efficacy of piglet vaccination***

In the experimental study (**section 3.1.**), significant differences were only obtained for the microscopic lung lesions, which were lower in group V1 (vaccinated three days before weaning) than in groups V2 (vaccinated at weaning) and PCG (non-vaccinated positive control group). In the field study (**section 3.2.**), significant differences were only obtained in one of the fattening

units where pigs were allocated, with group V1 having significantly higher weight gains and ADG than groups V2 and NV (non-vaccinated group) during the fattening period, as well as the whole study period. For the remaining efficacy parameters assessed in both studies, such as the prevalence and severity of macroscopic *Mycoplasma*-like lung lesions at necropsy or at slaughter, group V1 performed numerically better than group V2 and groups PCG or NV, though not statistically significantly. Overall, the aforementioned results implied a possible interference of the weaning process on the efficacy of *M. hyopneumoniae* vaccination. Nevertheless, the degree of this interference remains to be further elucidated in future studies.

The experimental challenge infection study described in this thesis was considered to be essential for the initial investigation of the effect of the weaning process on the efficacy of vaccination, since it allowed the selection of a highly virulent *M. hyopneumoniae* strain known to induce clinical disease, the use of a standard infection dose received by all animals and the avoidance of complications by other respiratory pathogens.

A possible reason for group V1 performing only numerically better than groups V2 and PCG for almost all parameters investigated in the experimental study of this thesis was that milder macroscopic lung lesions were observed compared to the previous experimental studies conducted by our research group (Meyns *et al*., 2006; Del Pozo Sacristán *et al*., 2012; Villarreal *et al*., 2012; Michiels *et al*., 2018). These studies used the same challenge strain and animal facilities. The reasons for the milder macroscopic lung lesions are not clear, but they could be attributed to the genetic background of the animals. In fact, the pigs used in the present study belonged to a different commercial hybrid line (Topigs 20) than the pigs used in the previous experimental studies of our research group (Rattlerow Seghers). Ruiz *et al*. (2002) found distinctive differences in the patterns of infection between pigs sired by different boars, with offspring from certain boars being able to clear out the pathogen faster after challenge infection. Although the role of sow genetics has not yet been assessed, the presence of the aforementioned differences concerning the

boar effect suggest that also sow genetics could be a source of variability for the observed macroscopic lung lesions between the present study and the previous experimental studies (Meyns *et al*., 2006; Del Pozo Sacristán *et al*., 2012a; Villarreal *et al*., 2012; Michiels *et al*., 2018).

The field study described in section 3.2. of this thesis allowed to include more animals in each treatment group, and to monitor PEP-induced clinical disease up to the time of slaughter. Additionally, it allowed to account for the management practices and environmental conditions that occurred under field conditions in the different production units that housed the age group employed. Thus, the beneficial effects of vaccinating three days prior to weaning on the growth parameters could be better investigated than in the experimental study. This is a possible explanation why group V1 performed significantly better than groups V2 and PCG in terms of weight gain and ADG in one of the fattening units housing pigs from the field study, whereas in the experimental study no significant differences were obtained among the different treatment groups. Another explanation is that experimental studies employ a lower number of animals (due to the present EU regulations concerning the use of experimental animals), and thus are not very well suited to assess possible differences among the different treatment groups on growth performance.

The herd employed in the current field study could be classified as 'positive – clinically affected' (Garza-Moreno *et al*., 2018). It presented an infection pattern that is typically found in many conventional farrow-to-finish operations, where a considerable percentage of the animals are already found infected in the late nursery period (Fano *et al*., 2007). In these operations, *M. hyopneumoniae*-induced 'active lung lesions' might heal by the time of slaughter, as there is considerable time between infection and slaughter. This might be one of the reasons why non-significant differences were obtained between group V1 and groups V2 and NV in terms of the prevalence and severity of macroscopic *Mycoplasma*-like lung lesions at slaughter. In fact, a high prevalence of fissures ('recovered lung lesions') was observed at slaughter, which was similar or

even higher than the prevalence of macroscopic *Mycoplasma*-like lung lesions observed across the different treatment groups. Another reason might be the number and virulence of the different *M. hyopneumoniae* strains involved, which were not known in this study. It has already been shown that low virulent strains cause less extensive and severe lung lesions than high virulent strains (Vicca *et al*., 2003; Meyns *et al*., 2007), and that a higher number of circulating *M. hyopneumoniae* strains in fattening pigs is associated with a higher prevalence and severity of macroscopic *Mycoplasma*-lung lesions at slaughter (Michiels *et al*., 2017).

Furthermore, the co-circulation of other respiratory pathogens might have influenced not only the prevalence and severity of macroscopic *Mycoplasma*-lung lesions in the current field study, but also the ability to acquire significant differences between treatment groups concerning the growth performance in both fattening units. Definitely, the SIV otubreak that occurred in the fattening unit that was within-site seemed to have influenced the evolution of respiratory disease, since after the outbreak that unit had significantly higher average RDS for the rest of the fattening period compared to the fattening unit that was offsite. Additionally, the co-circulation of other respiratory pathogens such as PRRSV and PCV-2 in both fattening units shows the existence of a typical PRDC situation, with these pathogens possibly playing an important role in the development of respiratory disease in the present herd. Given the potentiating or additive effect of *M. hyopneumoniae* to the aforementioned viral respiratory tract infections (Brockmeier *et al*., 2002; Thacker, 2002; Bochev, 2007), simultaneous vaccination and control measures against these pathogens might have enhanced the protection offered by *M. hyopneumoniae* vaccination against PRDC.

In both the experimental and the field study, a difference of only three days in the age of vaccination between groups V1 and V2 was chosen in order to ensure that in case of a disease outbreak (e.g. diarrhea due to enteric colibacillosis) the disease effect would be similar in both treatment groups, rather than having only one group vaccinated close to the disease outbreak.

Also, vaccinating group V1 at 18 days of age (i.e. three days before weaning), was considered to offer an adequate time for the adaptive immune responses to develop prior to the weaning process that occured on 21 days of age (Kick *et al*., 2011). For the pigs of group V2, it was speculated that the stressful events related to the weaning process would hinder the development of the adaptive immune responses following vaccination, by having a decreasing effect in the concentrations of $CD4^+$ and $CD8^+$ T-cells in the peripheral blood (Kick *et al*., 2012). This is the reason behind hypothesizing that the weaning process might have a negative effect on the efficacy of *M. hyopneumoniae* vaccination. These cells are implied to have an important role in the protection conferred by vaccination against PEP-induced clinical disease and lung lesions (Meyns *et al*., 2006; Villarreal *et al*., 2011; Marchioro *et al*., 2013). Humoral and cell-mediated immune responses are driven by activation of the $CD4^+$ T-cells (Stevens *et al*., 1988), while $CD8^+$ T-cells are able to suppress the *Mycoplasma*-induced pro-inflammatory responses (Jones and Simecka, 2003).

The same bacterin was used in both the experimental and field study. It was decided not to use other commercial bacterins, since this would complicate the comparison of the results between the experimental and the field study. Testing an additional bacterin in the same study would decrease the number of animals within each treatment group, thus decreasing the statistical power of the study. Especially for the experimental study, the committee that reviewed the ethical clearance application did not authorize the use of more animals.

Table 1 summarizes the results of other field studies utilizing Ingelvac MycoFLEX® in herds clinically affected with PEP**.** Baccaro *et al*. (2006) and Kaalberg *et al*. (2017) vaccinated the pigs at weaning, while Kristensen *et al*. (2014) chose to vaccinate the pigs during the first week of the nursery period. Variable results concerning the improvement of growth performance and also, the reduction of the prevalence and severity of *Mycoplasma*-like macroscopic lung lesions at slaughter were obtained between the vaccinated and the non-vaccinated treatment groups. This could be

attributed either to the irregular patterns of *M. hyopneumoniae* circulation in the selected herds (i.e. *M. hyopneumoniae* circulation peaking early during the nursery period or one month before slaughter) or to the simultaneous control of PCV-2 through vaccination (Table 1.).

Currently, there are no other published studies reporting on the effect of the weaning process on the efficacy of vaccination against *M. hyopneumoniae*. Overall, both sections 3.1. and 3.2. of this thesis highlight the complexity of studying the effects of *M. hyopneumoniae* vaccination protocols under experimental and field conditions. In the experimental study a limited number of animals was used, while in the field study only one herd was employed. Hence, the results of these studies implying a beneficial effect of vaccinating three days before weaning than at the day of weaning should be interpolated with caution when making vaccination protocols for endemically infected herds. In case of chosing to vaccinate three days before weaning, herd veterinarians are advised to evaluate the prevalence and severity of macroscopic *Mycoplasma*-like lung lesions across several production batches and perform an economic benefit evaluation analysis that includes growth performance in the parameters investigated (Maes *et al*., 2003).

**Table 1.** Field studies utilizing Ingelvac MycoFLEX® in pig herds clinically affected with PEP and published in international peer reviewed journals. Performance of the groups vaccinated with Ingelvac MycoFLEX® compared to the non-vaccinated (control) groups.

| Reference | Number of herds | Pigs vaccinated with Ingelvac MycoFLEX® | Increase in ADG g/pig/d (%) | Prevalence of *Mhyo*-like lesions at slaughter | Severity of *Mhyo*-like lesions at slaughter | Mortality | Comments |
|---|---|---|---|---|---|---|---|
| Baccaro *et al.* (2006) | 1 herd | 174/520 pigs | No siginficant effect | Significant reduction by 31.0% | Significant reduction by 67.0% | No significant effect | The short period between peak of *M. hyopneumoniae* circulation and slaughter could have contributed to the lack of significant differences in ADG. |
| Kristensen *et al*. (2014) | 2 herds | 740/2256 pigs | No siginficant effect | No significant effect | No significant effect | No significant effect | Lack of late *M. hyopneumoniae* circulation could have contributed to the non-significant effect of vaccination. |
| Kaalberg *et al*. (2017) | 1 herd | 267/812 pigs | Significant increase by 3% | Significant reduction by 41.0% | No significant effect | No significant effect (borderline; P=0.053) | Herd faced mixed *M. hyopneumoniae* and PCV-2 infections. The Ingelvac MycoFLEX® was injected together with Ingelvac CircoFLEX®. |

***Effect of gestating sow vaccination on the piglet colonization status during the peri-weaning period and the presence of Mycoplasma-like lung lesions at slaughter***

In the second field study (**section 3.3.**) of this thesis, in the first herd selected (herd A) that was already applying piglet vacination against *M. hyopneumoniae*, vaccination of the primiparous sows during late gestation significantly reduced the percentage of their piglets found to be colonized with *M. hyopneumoniae* at seven days post-weaning when compared to the piglets of the non-vaccinated sows. Additionally, piglets from vaccinated sows had a significantly reduced prevalence and severity of macroscopic *Mycoplasma*-like lung lesions at slaughter, than their counterparts from the non-vaccinated sows. In the second herd selected (herd B), which had a very low prevalence of *M. hyopneumoniae* colonization at weaning, there was no significant difference between the percentage of piglets originating from vaccinated and non-vaccinated sows that was found to be colonized at weaning and seven days post-weaning. Similarly, there were no significant differences detected between these pigs at slaughter, concerning the prevalence and severity of macroscopic *Mycoplasma*-like lung lesions observed. These results imply that in herds with a substantial percentage of piglets colonized with *M. hyopneumoniae* at weaning (e.g. herd A) compared to herds that have a low percentage of piglets colonized (e.g. herd B), the vaccination of primiparous gestating sows could further reduce the impact of *M. hyopneumoniae* infections in their piglets, when used in combination with piglet vaccination at weaning.

In the present study, there was no correlation between the colonization of the sows and the colonization of their piglets at weaning and seven days post-weaning. It seems that the predictability of the piglet colonization status based on the sow parity number alone may be misleading. In fact, *M. hyopneumoniae* is not only transmitted vertically from the sows to their piglets, but also horizontally between piglets sharing the same environment (e.g. farrowing unit) and belonging to different litters (during the suckling period) or pens (during the nursery period; Nathues *et al*., 2013). In the present study, there are numerous factors that might have played an

important role in enhancing horizontal transmission. These can be the colonization rates detected across the different sows present in the farrowing unit (Pieters *et al*., 2014), biosecurity procedures (Maes *et al*., 2008) and the single injectable iron dextran provision during the first days of age (Nathues *et al*., 2013). Additionally, the climate conditions in both the farrowing and the nursery units, may have played a role (Stärk *et al*., 2000; Nathues *et al*., 2013). Thus, the results of the present study suggest that horizontal transmission might have played an important role in the colonization rates detected among the piglets of the primiparous sows.

In the present field study, the levels of serum IgG antibodies elicited in primiparous sows after vaccination during late gestation correlated really well with the levels of serum IgG antibodies detected in their piglets. However, this is only an indirect proof that passive transfer of maternal immunity was enhanced in the piglets of the vaccinated primiparous sows and not that direct passive transfer of IgG antibodies from these sows was responsible for their piglets exhibiting lower colonization rates and improved respiratory health at slaughter (when compared to the piglets of the non-vaccinated primiparous sows). Given that it has been previously implied that maternally derived antibody-mediated immunity plays a non-significant role in protecting piglets from *M. hyopneumoniae* infection (Thacker *et al*., 2000), it can be hypothesized that other immunological components may have been involved in this protection.

As a result, further research is needed to determine which immunological components are responsible for conferring this resiliency to early *M. hyopneumoniae* colonization and the development of macroscopic *Mycoplasma*-like lung lesions during the later production stages in the piglets of the vaccinated sows when compared to the piglets of the non-vaccinated sows. Recent research is pointing towards the maternally derived cell-mediated immunity, which has been shown to participate in a functional response against the pathogen (Bandrick *et al*., 2008), and also to not interfere with the development of cell-mediated immune responses elicited in the piglets after them being vaccinated (Bandrick *et al*., 2014). This could be important under field

conditions, since in herds already applying *M. hyopneumoniae* piglet vaccination, the additional vaccination of the gestating sows could elicit beneficial anamnestic responses in their piglets that could further enhance protection against the pathogen when these piglets become infected (Rapp-Gabrielson *et al*., 2002; Martelli *et al*., 2006; Bandrick *et al*., 2014).

The reason why sow vaccination was performed twice in the present study (at six and three weeks before farrowing) was that the levels of *M. hyopneumoniae* IgG antibodies in the colostrum are comparable to these in the serum of the sows four weeks ante partum (Wallgren *et al*., 1998; Rautiainen and Wallgren, 2001). Then, until the time of partus the serum IgG antibody levels decrease, whereas the colostral IgG antibody levels increase. Hence, it was desired to achieve a high amount of maternal immunity transported to the colostrum by boosting the first vaccination with a second one. A second reason, is that literature proposes that sows farrowing are more likely to shed *M. hyopneumoniae*, due to the stressful nature of such procedure (Ruiz *et al*., 2003). Thus, it was desired to have the sows already immunized-boosted at a point that would presumably offer the best protection to their piglets relative to the time of farrowing.

In the present field study the sow vaccination protocol was alternately applied between four production batches. Additionally, the gilt replacement policies followed by both herds selected were not taking into account the *M. hyopneumoniae* infection status of the gilts purchased, neither included any specific acclimatization process during the quarantine period. It can be hypothesized that if this protocol is applied continuously between successive farrowing batches, and in herds with a proper gilt acclimatization process (Garza-Moreno *et al*., 2018), the results obtained would be further enhanced compared to the present field study.

Last but not least, Díaz *et al*. (2004) performed sow vaccination three weeks before farrowing, in addition to piglet vaccination, on a continuous pace over an eight-month period and found that in comparison with vaccinating only the piglets, it achieved a greater reduction in the prevalence of

pneumonic lesions at slaughter (15 *versus* 3%). Hence, it can be implied that continuous application of sow vaccination, in addition to piglet vaccination, in herds facing substantial early *M. hyopneumoniae* colonization levels would further stabilize herd immunity and reduce the impact of *M. hyopneumoniae* infections.

***Sampling methods used in this thesis for the detection of M. hyopneumoniae by PCR***

The sampling methods performed in the studies that focused on the effect of the weaning process on the efficacy of *M. hyopneumoniae* vaccination (**sections 3.1. and 3.2.**) were the collection of broncho-alveolar lavage fluid (BALF) and tracheobronchial swabs (TBS). They where chosen in terms of the ability to compare the detection rates of *M. hyopneumoniae* positive pigs with other experimental (Meyns *et al*., 2004; 2006; Villarreal *et al*., 2011; Del Pozo Sacristán *et al*., 2012a; Michiels *et al*., 2018) and field (Fablet *et al*., 2012; Del Pozo Sacristán *et al*., 2012b; 2014; Michiels *et al*., 2017) studies. BALF sampling is considered to be a very sensitive technique, offering similar detection rates to postmortem lung tissue sampling (Moorkamp *et al*., 2008). BALF can also be used for routine bacteriological culture to detect several bacterial respiratory pathogens (Villarreal *et al*., 2012). These were the reasons why it was preferred in the experimental study (**section 3.1.**). The TBS were preferred in the first field study of this thesis (**section 3.2.**), since they they are more practical to perform on a large number of animals under field conditions and also, for the fact that together with BALF they have been shown to offer better sensitivity for *M. hyopneumoniae* detection than nasal or tonsillar swabs (Kurth *et al*., 2002; Marois *et al*., 2007).

In the second field study of the current thesis (**section 3.3.**), the pigs were sampled by laryngeal swabs. Fablet *et al*. (2010) and Ruiz *et al.* (2003) suggested that the sensitivity of the sampling method in individual pigs can vary over time, depending on whether the exposure to the pathogen has been recent or chronic, and whether actual infection has occurred. Additionally, sampling

points from the lower respiratory tract are considered to offer better sensitivity than those from the upper respiratory tract (Kurth *et al*., 2002; Fablet *et al*., 2010). In contrast, Pieters *et al*. (2017) have shown that sampling from a tracheo-bronchial site did not offer better sensitivity compared to laryngeal swabs when real-time PCR was used as the method of detection in fattening pigs during the early stages of infection (between five and 28 weeks after experimental challenge infection). In the study described in section 3.3. of this thesis, it was decided to use laryngeal swabs as they were considered to be the most suitable method to conduct samplings in both sows and piglets, over the whole duration of this longitudinal study that employed a higher number of animals.

Although tracheo-bronchial and laryngeal sites are considered to be the most sensitive to detect the pathogen in weaned and finishing pigs (Takeuti *et al*., 2017), the larynx is not believed to be the normal habitat of the pathogen, and thus its presence in this site could either occur during the initial stages of the infection or when the pathogen is actively shed by the pig (Morris *et al*., 1994; Ruiz *et al*., 2003). In the study of Sibila *et al*. (2008) both of the NV and V sows were sampled two times; at seven weeks pre-farrowing and at one week post-farrowing. A decrease in the percentage of n-PCR positive sows was observed between the two sampling points, from 12 to 8% for the NV sows and from 28 to 0% for the V sows, further supporting the theory that shedding of the pathogen is of variable intensity and intermittent (Sibila *et al*., 2009; Maes *et al*., 2017). This intermittent shedding pattern can also be one of the reasons why in the second field study of this thesis (**section 3.3.**) the colonization rates observed in the piglets of herd A at seven days post-weaning were lower than those observed at weaning.

Last but not least, for both field studies described in this thesis (**sections 3.2. and 3.3.**), it was not possible to investigate the possibility of having more than one strains of *M. hyopneumoniae* circulating in the sampled pigs as there was no molecular typing performed in the *M. hyopneumoniae* qPCR or n-PCR positive samples obtained. A recent study by Michiels *et al*.

(2017) found that the prevalence and severity of *Mycoplasma*-like lung lesions at slaughter were significantly higher in batches of slaughter pigs where more different *M. hyopneumoniae* strains were found. Additionally, experimental studies have shown that after inoculation highly virulent strains were able to transmit faster and also, induce lung lesions and clinical disease earlier than the low virulent strains (Meyns *et al*., 2004; 2007; Villarreal *et al*., 2009). Thus, it could be that if more than one highly virulent *M. hyopneumoniae* strains circulated in the herds selected, the efficacy of *M. hyopneumoniae* vaccination in improving growth performance, and reducing the prevalence and severity of *Mycoplasma*-like pneumonia lesions at slaughter could have been impacted.

***General conclusions***

From the studies included in this thesis, the following conclusions can be drawn:

1) The vaccination of piglets against *M. hyopneumoniae* at three days before weaning when compared to vaccination at the day of weaning resulted in significantly less severe histopathological lung lesions, and in numerically lower prevalence and severity of macroscopic *Mycoplasma*-like lung lesions at necropsy or at slaughter. Additionally, taking into account both fattening units hosting pigs from the trial, numerically higher weight gains and average daily weight gains were obtained.
2) More herds with mixed respiratory disease where *M. hyopneumoniae* is involved as an important pathogen need to be investigated, in order to discover the construct decision trees for tailoring fattening pig vaccination protocols to the individual needs of each herd.
3) In a herd with a substiantial percentage of piglets colonized with *M. hyopneumoniae* at weaning, the vaccination of the primiparous sows during late gestation significantly reduced the percentage of their piglets found to be colonized with *M. hyopneumoniae* at seven days post-weaning when compared to the piglets of the non-vaccinated sows. Additionally, piglets from vaccinated sows had a significantly reduced prevalence and severity of macroscopic *Mycoplasma*-like lung lesions at slaughter, than their counterparts from the non-vaccinated sows.
4) In herds facing substantial early *M. hyopneumoniae* colonization levels, the vaccination of sows against *M. hyopneumoniae* during late gestation could be a useful tool to further stabilize the breeding sow herd immunity and subsequently, improve the transfer of colostral immunity to their progeny.

***Future research***

Further research on the effect of the weaning process on the efficacy of vaccination against *M. hyopneumoniae* should focus on selecting herds with different levels of *M. hyopneumoniae* infection, especially during the fattening period. In herds with a similar or higher infection level than the herd selected in the field study described in section 3.2., it would be interesting to modify the study protocol and adopt a different timing of vaccination relative to the day of weaning. For example, the pigs could be vaccinated after one week from their transfer to the nursery unit, instead of three days before weaning. By that way, vaccine-induced protective immunity will be possibly maintained at higher levels during the time period where in several herds PEP-induced clinical disease (i.e. coughing and growth retardation) reaches it peak, namely from the midth until the end of the fattening period (Vicca *et al*., 2002; Giacomini *et al*., 2016).

Apart from adopting a different timing of vaccination relative to the weaning process, more herds facing mixed respiratory tract infections with other respiratory pathogens that are primarily involved in PRDC (i.e. PRRSV, PCV-2, PRV or SIV) could be selected. This would allow to study between-pathogen interactions that have not been elucidated yet, and could possibly hinder the beneficial effect of vaccinating prior to weaning. Additionally, the possible effect of other vaccinations applied during the same time period on *M hyopneumoniae* vaccination or vice versa is not known.

In the field study described in section 3.3., an obvious reason behind the lower colonization rates and the improved slaughter performance in the pigs of the vaccinated sows could be the passive transfer (*via* suckling) of vaccine-induced maternal immunity. Nevertheless, the exact immunological components that are passively transferred and confer the aforementioned lower colonization rates and improved slaughter performance are not known. Over the last years, the focus is gradually shifting from the maternally derived antibody-mediated immunity (Thacker *et al*., 2000; Martelli *et al*., 2006) to the maternally derived cell-mediated immunity against *M.*

*hyopneumoniae* (Bandrick *et al*., 2008; 2011; 2014; Nechvatalova *et al*., 2011). It would be interesting to conduct future research to investigate how vaccine-induced colostral antigen-specific T-cell responses in newborn piglets correlate with the bacterial load in the lungs and the severity of PEP-induced lung lesions after challenge infection with a virulent *M. hyopneumoniae* strain.

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# Summary

**Summary**

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the primary agent of enzootic pneumonia, a chronic respiratory disease which results from infection of *M. hyopneumoniae* and other secondary bacteria, and inflicts major economic losses in the pig industry. One of the ways frequently used to control *M. hyopneumoniae* infections worldwide is vaccination. The most common vaccination strategy in practice is vaccination of the piglets during suckling or at weaning. Currently, it is not known whether the efficacy of vaccinating against *M. hyopneumoniae* can be influenced by the weaning process when vaccination is applied at the day of weaning.

Concerning the breeding sow population, vaccination of gestating sows against *M. hyopneumoniae* is not frequently practiced under field conditions. Nevertheless, breeding sows could be a reservoir of *M. hyopneumoniae* infections for the suckling and recently weaned piglets in endemically infected herds. Thus, it is interesting to investigate whether vaccinating sows during gestation could decrease the percentage of their offspring that is colonized with *M. hyopneumoniae* at weaning as well as in the nursery units.

The general aim of this thesis was to investigate different vaccination strategies against *M. hyopneumoniae* infections in order to improve the control of enzootic pneumonia.

The first study assessed the efficacy of a single *M. hyopneumoniae* vaccination (Ingelvac MycoFLEX®) three days before weaning (V1) or at weaning (V2) against experimental challenge infection. Four weeks after vaccination, groups V1 and V2 ($n$=20 pigs each) and a non-vaccinated, positive control group (PCG) ($n$=20) were inoculated endotracheally with a virulent *M. hyopneumoniae* strain. All pigs were euthanized five weeks after challenge. The average macroscopic lung lesion scores in groups V1, V2 and PCG were 0.54, 0.88 and 1.04, respectively ($P>0.05$). The average lymphohistiocytic infiltration scores in groups V1, V2 and PCG were 2.95, 3.16 and 3.61, respectively ($P<0.05$). The average qPCR values were: V1=$10^{2.94}$, V2=$10^{2.76}$ and

PCG=$10^{3.23}$ (P>0.05). Significant differences between groups V1 and V2 were only obtained for the histopathological lung lesions, where group V1 had a lower number of lesions.

In the second study, 828 piglets were randomly divided into three groups: group V1 was vaccinated three days before weaning, group V2 at weaning (21 days of age) and group NV was left non-vaccinated. Vaccinations were performed using Ingelvac MycoFLEX®. After the nursery period, 306 pigs were allocated to fattening unit (F1) and 501 pigs to a second unit (F2). Statistically significant differences were obtained in F2 where group V1 had a higher average daily weight gain compared to groups V2 and NV for the entire study period (17 and 18 g/day, respectively) and the fattening period (26 and 36 g/day, respectively) (P<0.05). Average lung lesion scores were: V1=3.44, V2=4.61, NV=4.55 (P>0.05), and prevalence of pneumonia: V1=35.0, V2=38.0, NV=41.4 (P>0.05). Overall, vaccination against *M. hyopneumoniae* ahead of weaning provided numerically better performance, but did not reach statistical significance.

The third study was performed in two herds. It investigated the effect of pre-farrowing primiparous sow vaccination (at six and three weeks before farrowing) against *M. hyopneumoniae* on offspring colonization at weaning and post-weaning, and lung lesions at slaughter. In each herd, two sow groups received *M. hyopneumoniae* vaccination with Ingelvac MycoFLEX® and two sow groups remained non-vaccinated. From each sow group, per herd, the litters of five primiparous sows were selected and sampled. Upon slaughter, the severity of *Mycoplasma*-like lung lesions (LLS) in these pigs was assessed. In herd A, 14.17% and 20.00% of the piglets from the vaccinated and non-vaccinated sows, respectively, were laryngeal swab-positive at weaning (P=0.225). At seven days post-weaning those values were 0.81% and 6.08%, respectively (P=0.031). In herd B, there were no statistically significant differences in the piglets from vaccinated and non-vaccinated sows that were laryngeal swab-positive at weaning (P=0.948) or seven days post-weaning (P=0.738). The average LLS in herd A was 15.54 for the piglets of the vaccinated sows and 26.40 for the piglets of the non-vaccinated sows (P=0.021). In herd B, those

values were 9.70 and 8.51, respectively (P=0.541). In conclusion, in herd A offspring from vaccinated sows had a significantly lower colonization rate seven days post-weaning and a significantly lower LLS at slaughter when compared to the offspring of the non-vaccinated sows.

From the studies included in this thesis, it was concluded that vaccination of piglets against *M. hyopneumoniae* prior to weaning when compared to their vaccination at the day of weaning conferred numerically, but not significantly better results across the majority of the efficacy parameters investigated. Additionally, in a herd with a substiantial early circulation of *M. hyopneumoniae*, the vaccination of primiparous gestating sows could further reduce the impact of *M. hyopneumoniae* infections in their piglets, when used in combination with early piglet vaccination.

Future research efforts on the effect of the weaning process on the efficacy of vaccination against *M. hyopneumoniae* should focus on selecting herds with different levels of *M. hyopneumoniae* infection, especially during the fattening period. Additionally, more herds facing mixed respiratory tract infections should be selected, in order to elucidate between-pathogen interactions that could possibly hinder the beneficial effect of vaccinating prior to weaning. Last but not least, it would be interesting to conduct future research to investigate whether vaccine-induced colostral antigen-specific T-cell responses in newborn piglets correlate with the bacterial load in the lungs and the severity of PEP-induced lung lesions after challenge infection with a virulent *M. hyopneumoniae* strain.

# Samenvatting

**Samenvatting**

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is het primaire agens van enzoötische pneumonie, een chronische ademhalingsziekte die het gevolg is van infecties met *M. hyopneumoniae* en andere secundaire bacteriën. De ziekte zorgt voor grote economische verliezen in de varkenshouderij. Vaccinatie is een controlemaatregel die wereldwijd vaak wordt gebruikt om *M. hyopneumoniae*-infecties te bestrijden. De meest toegepaste vaccinatiestrategie in de praktijk is vaccinatie van de biggen tijdens de lactatie of bij het spenen. Momenteel is niet bekend of de werkzaamheid van vaccinatie tegen *M. hyopneumoniae* nadelig wordt beïnvloed door de stress van het speenproces wanneer de vaccinatie wordt toegepast op de dag van spenen.

Wat de fokzeugpopulatie betreft, wordt vaccinatie van drachtige zeugen tegen *M. hyopneumoniae* niet vaak uitgevoerd onder veldomstandigheden. Niettemin vormen fokzeugen een reservoir van *M. hyopneumoniae*-infecties voor de biggen in endemisch geïnfecteerde bedrijven. Het is dus belangrijk om te onderzoeken of het vaccineren van zeugen tijdens de dracht het percentage biggen dat gekoloniseerd is met *M. hyopneumoniae* zowel bij het spenen als na het spenen kan verminderen.

De algemene doelstelling van dit proefschrift was om verschillende vaccinatiestrategieën tegen *M. hyopneumoniae* infecties te onderzoeken om de controle van enzoötische pneumonie te verbeteren.

De eerste studie beoordeelde de werkzaamheid van een one-shot *M. hyopneumoniae*-vaccinatie (Ingelvac MycoFLEX®) drie dagen voor het spenen (V1) of bij het spenen (V2) tegen een experimentele *M. hyopneumoniae* infectie. Vier weken na vaccinatie werden de groepen VI en V2 (*n*=20 varkens elk) en een niet-gevaccineerde positieve controlegroep (PCG) (*n*=20) endotracheaal geïnoculeerd met een virulente *M. hyopneumoniae*-stam. Alle varkens werden vijf weken na de infectie geëuthanaseerd en de longletsels werden beoordeeld. De gemiddelde macroscopische

longletselscores in de groepen V1, V2 en PCG waren respectievelijk 0,54, 0,88 en 1,04 (P>0,05). De gemiddelde lymfohistiocytaire infiltratiescores in de groepen V1, V2 en PCG waren respectievelijk 2,95, 3,16 en 3,61 (P <0,05). De gemiddelde qPCR-waarden waren: V1=$10^{2,94}$, V2=$10^{2,76}$ en PCG=$10^{3,23}$ (P>0,05). Significante verschillen tussen groepen V1 en V2 werden alleen verkregen voor de histopathologische longletsels, waarbij groep V1 minder uitgebreide laesies had.

In het tweede onderzoek werden 828 biggen willekeurig verdeeld in drie groepen: groep V1 werd drie dagen vóór het spenen gevaccineerd, groep V2 bij spenen (21 dagen oud) en groep NV werd niet gevaccineerd. Voor de vaccinaties werd het vaccin Ingelvac MycoFLEX® gebruikt. Na de biggenbatterijperiode werden 306 varkens toegewezen aan een mestafdeling (F1) en 501 varkens aan een tweede mestafdeling (F2). Statistisch significante verschillen werden verkregen in F2, waar groep V1 een hogere gemiddelde dagelijkse gewichtstoename had in vergelijking met groepen V2 en NV voor de gehele onderzoeksperiode (respectievelijk 17 en 18 g/dag) en voor de afmestperiode (26 en 36 g/dag, respectievelijk) (P<0,05). Gemiddelde longletselscores waren: V1=3,44, V2=4,61, NV=4,55 (P>0,05) en prevalentie van pneumonie: V1=35,0, V2=38,0, NV=41,4 (P> 0,05). Over het algemeen leidde vaccinatie tegen *M. hyopneumoniae* vóór het spenen numeriek tot betere prestaties, maar de verschillen waren niet statistisch significant.

De derde studie werd uitgevoerd in twee varkensbedrijven. Het effect van vaccinatie tegen *M. hyopneumoniae* van primipare zeugen op het einde van de dracht (zes en drie weken voor het werpen) op de kolonisatie van *M. hyopneumoniae* bij de biggen bij en na het spenen en op het voorkomen van longletsels op slachtleeftijd werd nagegaan. In elk bedrijf werden twee zeugengroepen gevaccineerd tegen *M. hyopneumoniae* met Ingelvac MycoFLEX® en werden twee zeugengroepen niet gevaccineerd. Van elke zeugengroep binnen elk bedrijf werden de tomen van vijf primipare zeugen geselecteerd en bemonsterd. Na het slachten werd de ernst van de Mycoplasma-like longletsels (LLS) bij deze varkens beoordeeld. In bedrijf A testten 14,17% en

20,00% van de biggen van de respectievelijk gevaccineerde en niet-gevaccineerde zeugen positief d.m..v. laryngaal swabs bij het spenen (P=0,225). Zeven dagen na het spenen waren die percentages respectievelijk 0,81% en 6,08% (P=0,031). In bedrijf B waren er geen statistisch significante verschillen tussen de biggen van gevaccineerde en niet-gevaccineerde zeugen op basis van laryngeaal swab-positief zijn voor *M. hyopneumoniae* bij het spenen (P=0,948) of zeven dagen na het spenen (P=0,738). De gemiddelde LLS in bedrijf A was 15,54 voor de biggen van de gevaccineerde zeugen en 26,40 voor de biggen van de niet-gevaccineerde zeugen (P=0,021). In bedrijf B waren die waarden respectievelijk 9,70 en 8,51 (P=0,541). Concluderend, in bedrijf A hadden nakomelingen van gevaccineerde zeugen een significant lagere kolonisatiegraad zeven dagen na het spenen en een significant lagere LLS bij het slachten in vergelijking met de biggen van de niet-gevaccineerde zeugen.

Uit deze studies opgenomen in dit proefschrift, werd geconcludeerd dat vaccinatie van biggen tegen *M. hyopneumoniae* voorafgaand aan het spenen in vergelijking met vaccinatie op de dag van het spenen tot numeriek (maar niet statistisch significant) betere resultaten leidde voor het grootste deel van de onderzochte werkzaamheidsparameters. Bovendien, zou de vaccinatie van drachtige eersteworpszeugen in een bedrijf met een substantieel vroege circulatie van *M. hyopneumoniae*, de impact van *M. hyopneumoniae*-infecties bij hun biggen verder kunnen verminderen, indien toegepast in combinatie met vroege biggenvaccinatie.

Toekomstig onderzoek naar het effect van het speenproces op de werkzaamheid van vaccinatie tegen *M. hyopneumoniae* moet zich concentreren op het selecteren van bedrijven met verschillende niveaus van *M. hyopneumoniae*-infectie, vooral tijdens de mestperiode. Bovendien moeten meer bedrijven worden geselecteerd waarbij er menginfecties van de luchtwegen optreden, om een eventueel negatief effect op *M. hyopneumoniae* vaccinatie te onderzoeken. Tenslotte, zou het interessant zijn om te onderzoeken of door vaccin geïnduceerde colostrale antigeen-specifieke T-celreacties bij pasgeboren biggen correleren met het aantal kiemen in de longen en de ernst van

*Mycoplasma*-geïnduceerde longletsels na challenge-infectie met een virulente *M. hyopneumoniae*-stam.

# *Curriculum vitae*

***Curriculum vitae***

**Ioannis Arsenakis** was born on December 11th 1984 in Chicago, USA. He graduated in March 2010 from the Faculty of Veterinary Medicine of Aristotle University of Thessaloniki, Greece. He then received a scholarship grant to continue his studies at the Roslin Institute of the Royal (Dick) School of Veterinary Studies in Edinburgh, Scotland. He graduated on June 2012 and received a Master's degree in Animal Biosciences (awarded with Distinction). He then continued in the Republic of Ireland, where he worked as a poultry veterinarian, taking care of broiler and broiler breeder flocks. From August 2013 until October 2018, he continued working as a swine clinician at the Unit of Porcine Health Management of the Faculty of Veterinary Medicine of Ghent University, Belgium. He completed the Residency Program for the European College of Porcine Health Management and also performed several clinical studies. These studies mainly focused on the optimization of the efficacy of vaccination strategies against *M. hyopneumoniae* in peri-weaned piglets and breeding sows. Other studies he conducted and published focused on the use of autogenous vaccination in sows for the control of exudative epidermitis in nursery pigs and the evaluation of semen quality of Belgian Piétrain boars. Additionally to the above, Ioannis is interested in all aspects of swine health and production. His practical work involved a wide area of clinical practice, by mainly providing second opinion technical advice in swine herds facing health and production issues as well as artificial insemination centers facing fertility issues. He has been an invited speaker on several national and international conferences, and a first author or co-author of several papers in international peer reviewed scientific journals.

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