Epidemiology of *M. hyopneumoniae* infections and effect of control measures

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AIAO          All-in/ all-out
ANOVA         Analysis of variance
ADG           Average daily weight gain
BALF          Bronchoalveolar lavage fluid
BALT          Bronchus-associated lymphoid tissue
CCU           Color changing units
DPI           Days post infection
EP            Enzootic pneumonia
ELISA         Enzyme-linked immunosorbent assay
EU            European Union
ELI           Expression library immunization
HPLC-H₂O      High performance liquid chromatography water
HV            Highly virulent
H₂O₂          Hydrogen peroxide
IF            Immunofluorescence
Ig            Immunoglobulin
IFN-γ         Interferon gamma
IL            Interleukin
LV            Low virulent
nPCR          Nested polymerase chain reaction
NrdF          Ribonucleotide reductase
NV            Non vaccinated
OD            Optical density
PBS           Phosphate buffered saline
PCR           Polymerase chain reaction
PCV2          Porcine circovirus type 2
PRCV          Porcine respiratory corona virus
PRDC          Porcine respiratory disease complex
PGE2          Prostaglandin E
RDS           Respiratory disease score
R₀            Adjusted reproduction Ratio
PRSSV         Porcine reproductive and respiratory syndrome virus
SPES          Slaughterhouse Pleurisy Evaluation System
SD            Standard deviation
S-I           Susceptible-infectious
SIV           Swine influenza virus
Th            T helper cells
TNF-α         Tumor necrosis factor alfa
V             Vaccinated
w             Weeks
CHAPTER 1.

GENERAL INTRODUCTION
1.1. LITERATURE REVIEW

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the primary etiological agent of enzootic pneumonia (EP), one of the most prevalent respiratory diseases in pigs (Thacker, 2006). In field situations, it acts as a door opener for secondary bacterial pathogens. *M. hyopneumoniae* is one of the main pathogens involved in the porcine respiratory disease complex (PRDC) (Opriessnig et al., 2004; Thacker, 2006). PRDC is often caused by *M. hyopneumoniae*, accompanied by infections with secondary bacteria such as *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, *Pasteurella multocida*, *Streptococcus suis*, as well as with viral agents such as porcine reproductive and respiratory syndrome virus (PRSSV), porcine respiratory corona virus (PRCV), swine influenza virus (SIV), and porcine circovirus type 2 (PCV2). EP and PRDC inflict worldwide economic losses in the swine industry (Ross, 1999; Rautiainen et al., 2000; Georgakis et al., 2002; Maes et al., 2008) due to decreased growth rate, increased feed conversion ratio, increased treatment costs and increased mortality, emphasizing the importance of *M. hyopneumoniae* among the scientific community.

This review aims to discuss the most recent findings on the following aspects of *M. hyopneumoniae* infections and/or enzootic pneumonia: the aetiology and pathogenesis, the epidemiology of *M. hyopneumoniae* infections with special emphasis on transmission, differences between low and highly virulent *M. hyopneumoniae* strains, and finally strategies for control with focus on vaccination.

### 1.1.1. Aetiology

*M. hyopneumoniae* belongs to the mollicutes taxon and ranks among the smallest (0.2 µm) self-replicating bacteria known to date (Tajima and Yagihashi, 1982; Jacques et al., 1992; Blanchard et al., 1992). It has a very small and simple genome (893 - 920 kilo-base pairs), and the organism
belongs to the most sequenced porcine mycoplasmas (strains J, 232 and 7448) (Minion et al., 2004; Vasconcelos et al., 2005). *M. hyopneumoniae* is extremely sensitive to environmental conditions and in contrast to most prokaryotic organisms, it is characterized by the lack of a cellular wall. The pathogen is not able to survive for a long time outside its host, but in aerosols its survival time increases as it can remain infective for up to 31 days in water at 2-7°C (Goodwin, 1972).

The organism is characterized by a slow and fastidious growth *in vitro* and is extremely difficult to isolate (Friis, 1974). Therefore, isolation is not used for routine diagnosis. The first isolations of *M. hyopneumoniae* were done from pneumonic lung tissue and took place only four decades ago (Goodwin et al., 1965; Maré and Switzer, 1965). The J strain was isolated in 1973 (Whittlestone, 1973). It has become a high passage laboratory strain that is considered as the reference strain for *M. hyopneumoniae*. The fastidious growth is related to its dependence on the supply of nutrients and the need for constant environmental conditions. Therefore, a wide spectrum of essential amino acids and fatty acids provided by either the host or the culture medium is required, since a considerable proportion of genes involved in cofactor biosynthesis has been lost throughout its evolution (Razin et al., 1998; Pollack, 2002).

A parasitic way of life has been achieved through the manifestation of surface proteins (Chambaud et al., 1999). The organism can produce adhesins, modulins, aggressins and impedins that allow adhesion and modulation of the host immune system (Asai et al., 1996; Henderson et al., 1996; Muneta et al., 2006, 2008). Using molecular techniques, it appears that *M. hyopneumoniae* isolates show a large diversity at genomic (Stakenborg et al., 2006; Major et al., 2007; Strait et al., 2008) and proteomic (Calus et al., 2007) level. In addition, there are also important differences in virulence among *M. hyopneumoniae* isolates (Vicca et al., 2003; Strait et
al., 2004). Based on respiratory disease score, lung lesions scores, histopathology, immunofluorescence and serology, three groups of isolates were obtained, namely low, moderate and highly virulent ones (Vicca et al., 2003).

1.1.2. Pathogenesis

*M. hyopneumoniae* is a host specific bacterium which only infects pigs. The organism attaches to cilia and colonizes the mucosal surface of the ciliated epithelium of the trachea, bronchi and bronchioles of pigs (Blanchard et al., 1992; Kwon et al., 2002; Saradell et al., 2003). Adhesion is a prerequisite for initiation of disease (Tajima and Yagihashi, 1982). It is a process mediated by carbohydrates and proteins (Zielinski et al., 1993; Li et al., 2009) present on this microorganism’s surface and the ciliated epithelium of the respiratory tract of the pig. Although adhesion to cilia is important to cause pneumonia, differences in virulence between strains were not associated with their adhesion capacity *in vitro* (Calus et al., 2009). Some *Mycoplasma* species (*e.g.* *M. gallisepticum*) have the ability to penetrate and live within host cells (Winner et al., 2000), but there is no such evidence for *M. hyopneumoniae* (Jenkins et al., 2006).

*M. hyopneumoniae* infections interfere with the normal functioning of the cilia in the respiratory tract, affecting the nonspecific defense mechanisms. As a result, infected animals are more susceptible to secondary bacterial infections (Ciprian et al., 1988; Sørensen et al., 1997). The ciliated epithelial cells of the trachea, the bronchi and bronchioles, the secretion of mucus and the alveolar macrophages are important for removing noxious inhaled particles and pathogens from the respiratory system. Once the ciliary activity is impaired, the mucosal clearance system becomes ineffective, leading to hypersecretion and evident presence of exudate caused by an altered glycoprotein production in goblet cells (DeBey et al., 1992). The mechanism behind cilia
damage is not fully understood, but it has been suggested that an increased inflow of calcium ions into the epithelial cells (DeBey et al., 1993; Park et al., 2002) may result in ciliostasis and gradual loss of cilia. A membrane-associated cytopathic factor (54 kDa protein) has been found to induce cytotoxicity to tracheal rings, but its role remains unclear (DeBey and Ross, 1994). For some mycoplasmas (*e.g.* *M. mycoides*) (Miles et al., 1991; Rice et al., 2001), it has been suggested that the production of hydrogen peroxide (H$_2$O$_2$) might be involved in the damage of host cells. It is not known whether this is also the case for *M. hyopneumoniae*. The damage of the epithelium in the respiratory tract and the infiltration of exudate often facilitate the entry of other respiratory pathogens such as *P. multocida*, *A. pleuropneumoniae*, *B. bronchiseptica*, among many others, that can considerably aggravate the condition of the infected pig.

In addition, the ability of *M. hyopneumoniae* to modulate the host immune response enables it to evade or suppress the pig’s defense mechanisms and establish a chronic, persistent infection (Razin et al., 1998). Studies have shown that the organism remains in the lungs until at least 6 (Fano et al., 2005a) or 8 months (Pieters et al., 2009) after challenge infection. It has been demonstrated that phagocytosis by alveolar macrophages is suppressed during *M. hyopneumoniae* infection (Caruso and Ross, 1990). Moreover, Asai et al. (1996) demonstrated that the chemiluminescence response in neutrophils obtained from broncho-alveolar lavage (BAL) fluid in pigs experimentally infected with *M. hyopneumoniae* was suppressed, indicating a decrease in the function of these cells.

The mechanisms involved in the pathogenicity of *M. hyopneumoniae* remain unclear. A remarkable increase in the number of neutrophils and higher titres of pro-inflammatory cytokines TNF-$\alpha$ and IL-1 in BAL fluid were detected in pigs infected with a highly virulent strain compared to those infected with low virulent ones at 10, 15 and 28 DPI (Meyns et al., 2007).
faster *in vitro* multiplication of the highly virulent isolate during the logarithmic phase has also been described (Calus et al., 2010), suggesting a higher capacity to multiply in the lungs. Further research is ongoing to compare the colonization characteristics of highly and low virulent strains.

### 1.1.3. Occurrence and epidemiology of *M. hyopneumoniae* infections

*M. hyopneumoniae* has been detected in almost all pig farms in countries with intensive pig production (Furlong et al., 1975; Madec and Kobisch, 1982; Rautiainen et al., 2001; Sibila et al., 2004a; Maes et al., 2008; Silva et al., 2009). Table 1 shows the occurrence of infection in pigs of different age groups reported in various studies.
<table>
<thead>
<tr>
<th>Occurrence of <em>M. hyopneumoniae</em></th>
<th>nPCR (nasal swabs)</th>
<th>nPCR (bronchial/tracheal swabs)</th>
<th>nPCR (BALF)</th>
<th>Serology</th>
<th>Author(s)</th>
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<tbody>
<tr>
<td><strong>Suckling pigs</strong></td>
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<td></td>
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<tr>
<td>13.0%</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>Palzer, 2006</td>
</tr>
<tr>
<td>1.5-3.8%</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Sibila et al., 2007a</td>
</tr>
<tr>
<td>0.5-2.1%</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Sibila et al., 2007b</td>
</tr>
<tr>
<td>12.3%</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>Moorkamp et al., 2009</td>
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<tr>
<td><strong>Weaned pigs</strong></td>
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<tr>
<td>10.0-60.0%</td>
<td>+</td>
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<td></td>
<td>Calsamiglia et al., 1999</td>
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<tr>
<td>1.3-13.1%</td>
<td>+</td>
<td></td>
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<td></td>
<td>Ruiz et al., 2003</td>
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<tr>
<td>10.1%</td>
<td>+</td>
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<td></td>
<td>Sibila et al., 2004</td>
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<tr>
<td>0.0-51.2%</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Fano et al., 2007</td>
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<tr>
<td>2.8-7.8%</td>
<td>+</td>
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<td>Sibila et al., 2007b</td>
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<tr>
<td>3.0-51.0%</td>
<td></td>
<td>+</td>
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<td></td>
<td>Sibila et al., 2008</td>
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<tr>
<td>10.6%</td>
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<td>+</td>
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<td></td>
<td>Moorkamp et al., 2009</td>
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<tr>
<td><strong>Fattening pigs at slaughter</strong></td>
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<tr>
<td>19.0%</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>Morris et al., 1995</td>
</tr>
<tr>
<td>0.0-100%</td>
<td>+</td>
<td></td>
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<td></td>
<td>Maes et al., 1998</td>
</tr>
<tr>
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<td></td>
<td>Maes et al., 1999</td>
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<td>Rautiainen et al., 2000</td>
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<td>8.0-100%</td>
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<td>Fano et al., 2007</td>
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<tr>
<td>29.3-61.1%</td>
<td>+</td>
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<td></td>
<td>Sibila et al., 2007b</td>
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<tr>
<td>79.0-86.0%</td>
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<td>+</td>
<td></td>
<td></td>
<td>Sibila et al., 2007b</td>
</tr>
<tr>
<td><strong>Sows</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>24.0-56.0%</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Calsamiglia, 2000</td>
</tr>
<tr>
<td>27.5%</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Sibila et al., 2007a</td>
</tr>
<tr>
<td>33.3%</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Sibila et al., 2007b</td>
</tr>
<tr>
<td>24.0-36.0%</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Sibila et al., 2008</td>
</tr>
<tr>
<td>65.0%</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>grosse Beilage et al., 2009</td>
</tr>
</tbody>
</table>

**Suckling and weaned pigs**

The percentage of suckling pigs positive for *M. hyopneumoniae* by nPCR in BAL fluid among the suckling population (1-3 weeks of age) in two German studies was approximately 10% (Moorkamp et al., 2009; Palzer, 2006). A similar but slightly higher percentage of positive pigs
by nPCR in BAL fluid was found in weaned pigs (3-6 weeks of age) in Germany (Moorkamp et al., 2009) and Spain (Sibila et al., 2004; Sibila et al., 2008). In all these studies, the selected herds had a history of respiratory disease. Under field conditions, there is a progressive decrease in the numbers of pigs with maternal antibodies followed by a slow increase in the numbers of seropositive animals towards the end of the nursery stage (Calsamiglia et al., 1999b). The prevalence of *M. hyopneumoniae* based on nPCR testing on nasal swabs in weaned pigs has been suggested as a potential indicator of shedding by sows (Ruiz et al., 2003) and a possible predictor of the severity of enzootic pneumonia in older animals (Fano et al., 2007; Sibila et al., 2007a; and Sibila et al., 2007b).

**Fattening pigs**

The percentage of animals with antibodies against *M. hyopneumoniae* at slaughter ranges from 0 to 100% (Maes, 1998). However, the prevalence largely varies between farms, production systems and countries. Maes et al. (1999) investigated epidemiological aspects in slaughter pigs from 50 fattening pig herds, reporting that the seroprevalence at herd level for *M. hyopneumoniae* was 88%. Rautiainen et al. (2000) reported that seroprevalences fluctuated between 54 and 81% in fattening pigs. Detection in bronchial (or tracheal) swabs by nPCR ranges between 79-86% (Sibila et al., 2007b) and up to 100% (Fano et al., 2007).

**Sows**

*M. hyopneumoniae* seroprevalence in sows varies widely between studies namely from 0 to 56% (Calsamiglia and Tijooan 2000; Sibila et al., 2007). A cross-sectional study carried out in 2578 sows from 67 herds in north-west Germany by grosse Beilage et al. (2009) showed that 65% of the sows were seropositive.
Despite the common practice of vaccination and its beneficial effects against EP in pigs, the overall occurrence of this pathogen is not significantly different in vaccinated and non-vaccinated animals (Meyns et al., 2006; Maes et al., 2008).

1.1.3.1. Transmission of *Mycoplasma hyopneumoniae*

Within a herd, *M. hyopneumoniae* can be transmitted by both vertical and horizontal transmission (Ross, 1999; Thacker, 2006; Fano et al., 2007). Vertical transmission is the transfer of a pathogen from a parent (usually the mother) to the offspring. Horizontal transmission is the transfer of a pathogen from an infected animal to a naïve animal, independent of the parental relationship of those individuals. Horizontal transmission of *M. hyopneumoniae* may occur by either direct nose-to-nose contact with infected pigs or indirectly by sharing the same air space with infected animals. Airborne transmission is considered a form of indirect horizontal transmission by which pathogenic agents contained in aerosols spread from infected to non-infected susceptible individuals (Kurkjian et al., 2003). Introduction of infected animals and airborne transmission (Cardona et al., 2005) are the two main sources for introduction of *M. hyopneumoniae* in a herd (Ross, 1999).

**Sow to piglet transmission**

Despite the limited existence of studies on sow-to-piglet transmission, it is generally accepted that the sow plays an important role in transmitting the pathogen to the offspring, and consequently, to maintain the infection within a herd (Sibila et al., 2007; grosse Beilage et al., 2009). Transmission occurs when the sow has direct nose-to-nose contact with her suckling piglets (Rautiainen and Wallgren, 2001; Thacker et al., 2006), since no *in-utero* or lactogenic transmission has been documented (Burgi, 1990; Maes et al., 1998). Sibila et al. (2007)
demonstrated that *M. hyopneumoniae* can be detected in the lower and upper respiratory tract of piglets as early as between the 1\textsuperscript{st} and 3\textsuperscript{rd} week of age.

Several studies have shown that younger sows are more likely to transmit the infection to the offspring (Goodwin 1965; Morris et al., 1994; Sibila et al., 2007). Fano et al. (2006) showed that the prevalence at weaning in piglets from young sows (parity 1-2) was 1.6 higher than in piglets from older sows (parity > 5). Hence, the risk of being infected or tested positive decreases with parity or age of the sow. Partial depopulation in a herd (removing pigs younger than 10 months of age and leaving the older sows) has been used as an alternative in the past for a successful eradication protocol in Switzerland (Zimmermann et al., 1989). The program was shown to be successful to eliminate *M. hyopneumoniae* in small pig herds. It is currently seldom used in larger pig herds.

These findings also correspond with those of a recent cross-sectional study involving 2578 sows from 67 herds in Germany, in which a negative correlation was found between seropositivity in sows and their parity (grosse Beilage et al., 2009). Although older sows (third to seventh parity) are less likely to transmit *M. hyopneumoniae* to their offspring, they also retain the potential to spread *M. hyopneumoniae* to their piglets (Calsamiglia and Pijoan, 2000).

**Horizontal transmission**

Horizontal transmission can occur by direct nose-to-nose contact (Done, 1996) with respiratory secretions from infected animals or water droplets from coughing pigs to susceptible animals, or by indirect airborne transmission (Goodwin, 1985; Stärk et al., 1998a; Dee et al., 2009).

a. **Direct contact**
Nose-to-nose transmission can occur from infected to susceptible animals in all age groups. (Clark et al., 1991). Pigs from different pens can infect each other by direct contact if no solid pen partitions are present. Based upon findings of an experimental transmission study, pigs infected with *M. hyopneumoniae* will infect on average one penmate during the nursery period (Meyns et al., 2004), indicating that the spread of this pathogen is slow (Batista et al., 2004; Fano et al., 2005a; Meyns et al., 2006). Piglets between 3 and 12 weeks of age seem to be equally susceptible to this pathogen (Piffer et al., 1984). Once the pathogen has been introduced and settled in the herd, it continues to spread to other age groups and infection is maintained within the farm.

Since parameters like temperature and humidity are influenced by outdoor weather conditions, infection and reinfection are more likely to occur in autumn and winter (Goodwin et al., 1985; Jorsal et al., 1988; Thomsen et al., 1992). It has been observed that clinical signs of mycoplasmal disease in pigs reach their peak between November and March (Stärk, 1992), due to the pathogen’s ability to survive in cold and wet conditions. One of the first authors to investigate the influence of temperature and humidity on enzootic pneumonia was Gordon (1963). He concluded that warmer environmental conditions had a beneficial effect on respiratory diseases in pigs. Some years later, Whittlestone (1976) also showed experimental evidence of the effect of temperature and humidity on the survival of *M. hyopneumoniae* by demonstrating an inverse relationship between enzootic pneumonia and temperature increase in dry conditions. Finally, Geers et al. (1989) observed a negative correlation between coughing and air temperature.

Multi-source purchase policy is considered as one of the most important factors in the introduction of respiratory agents by infected animals into a farm (Rosendal and Mitchell, 1983; Thomsen et al., 1992; Hurnik et al., 1994; Stärk, 2000; Rautiainen, 2001). Hege et al. (2002)
reported that pig farmers who bought animals from only one supplier per batch had a significantly lower risk for introduction of this pathogen than farmers with other purchasing practices.

On the other hand, littermates can retain the pathogen in their lungs without displaying clinical symptoms (Goodwin, 1965). Consequently, the likelihood of introduction of infected animals increases when purchased pigs remain carriers of the organism without displaying symptoms. Infected animals may carry *M. hyopneumoniae* bacteria in the lungs even up to six months post infection (Fano et al., 2005a).

The transmission of two different *M. hyopneumoniae* strains in nursery piglets was first quantified by estimating a transmission ratio six years ago (Meyns et al., 2004). In that experimental study, infected seeder pigs were placed together with susceptible animals during 6 weeks and the number of contact-infected animals was determined at the end of the study, using Rn-values. The transmission ratios (Rn-value)s of the highly virulent (1.47) and the low virulent isolates (0.85) were not significantly different, whereas the overall Rn was estimated to be 1.16. It was concluded that one *M. hyopneumoniae* infected animal at weaning will infect on average one penmate during the nursery period.

b. **Indirect contact**

   o **Airborne Transmission**

Regarding airborne transmission, not only on-farm infected pigs can be a source of infection, but also infected pigs from neighboring herds. The first successful detection of airborne *M. hyopneumoniae* under experimental and field conditions with a PCR assay was performed in Switzerland (Stärk et al., 1998a).
The distance to the closest infected farm, its size and the pig density are important risk factors for infection with *M. hyopneumoniae* (Dohoo et al., 2009; Stärk, 2000). The risk factors for infection with this pathogen are presented in Table 2, p.32. In diseased herds, sneezing and coughing animals generate large amounts of airborne particles, which can potentially cause infection in susceptible animals. According to Hirst (1995), aerosols are defined as solid or liquid particles suspended in air. The concentration of infectious agents in aerosols is proportional to the number and concentration of infected animals on a farm (stocking density, herd size) or a region (pig density) (Stärk et al., 1998b).

Goodwin (1985) stated that 3.2 km is the minimal prudent distance at which non infected herds should be situated from *M. hyopneumoniae* positive herds. Vicinity to infected herds within 2 to 3 km is a risk factor for the aerogen transmission between herds (Thomson et al., 1992). However, more detailed studies need to be performed in this regard, since the most recent data have shown that long-distance airborne transmission of this pathogen can occur at a much longer distance (Dee et al., 2009). *M. hyopneumoniae*-positive samples have been recovered from air samples up to 9.2 km from the herd of origin (Otake et al., 2010), and these samples were still infectious.

The influence of environmental conditions and seasonal patterns also seem to have an effect on the airborne spread of *M. hyopneumoniae* (see Table 2, p. 32), although the causal relationship between environmental risk factors and respiratory diseases is rather complex (Stärk, 2000).

The details of airborne survival for many bacterial species have not been studied in detail. It is known, however, that radiation, sun light, air ions, relative humidity and pollutants affect the biological decay of aerosolised agents. The sensitivity to some of these factors is related to the lipid surface composition of a microorganism. *M. hyopneumoniae* and other mycoplasmas have a
large number of prominent lipoproteins on the surface membrane (Chambaud et al., 1999; Ferreira et al., 2007), which appears to have a marked influence on their survival at low temperatures (Raccach et al., 1975). In fact, micro-organisms generally survive better at low temperatures (Heber et al., 1988; Butera et al., 1991). Such resistance to open air factors is important for this pathogen to remain infective, even when spread over long-distances. A study about infectious aerosols in pig disease transmission (Stärk, 1998b) states that long distance transport and survival of airborne microorganisms in pigs seems to be favored by damp, cool, dark conditions, and in areas with flat topography (free of vegetation) and stable atmospheric conditions. All these aforementioned conditions seem to favor long distance transmission of \textit{M. hypneumoniae}.

- **Mechanical vectors**

The role of personnel and fomites as mechanical vectors for the transmission of \textit{M. hyopneumoniae} between pigs or between farms has not been clearly determined (Done, 1996; Amass et al., 2000; Batista et al., 2004) and is considered to be of limited importance. Hege et al. (2002) reported that a parking site for pig transport vehicles situated 10 to 200 m to the farm was one of the main risk factors for re-infection of herds with \textit{M. hyopneumoniae}. In contrast to these findings, Batista et al. (2004) reported that fomites were not important for the transmission of \textit{M. hyopneumoniae}. Further studies are necessary to clarify the role of equipment and personnel in the transmission of \textit{M hyopneumoniae}. 
1.1.4. Risk factors for infection with M. hyopneumoniae in pig herds

The most important risk factors for infection with M. hyopneumoniae are related to herd characteristics, management practices and housing conditions, biosecurity measures and seasonal effects. A list of the major risk factors associated with the risk for M. hyopneumoniae infections and / or respiratory disease and published in literature, is presented in the control section of this introduction (see further Table 2, p. 32).

Herd size and herd type are factors among herd characteristics found to influence the incidence of enzootic pneumonia in pigs (Goodwin et al., 1985; Stark et al., 2000; Maes et al., 2000). In general, there is a positive association between increasing herd size and the presence of M. hyopneumoniae infections in the herd.

Different purchase policies are also reflected in different herd types. For instance, growing-finishing farms are more dependent on animal supply than breeding farms are, which makes them more likely to buy large numbers of animals from different sources (Hofer et al., 2002; Stark et al., 2000). A multi-source purchase policy largely contributes to introduction of clinically or sub-clinically infected animals that can easily spread the pathogen among susceptible pigs (Thomsen et al. 1992; Hege et al., 2002).

The clinical outcome of M. hyopneumoniae infection is also dependent on environmental and management conditions and on the production system in operation. For instance, a continuous flow of pigs through facilities increases the odds of infection. Production systems where pigs are moved through the different production stages in batches (all-in/all-out), as opposed to continuous flow systems, generally include cleaning and disinfecting steps which reduce micro-organism concentrations (Stark et al., 2000). In most reviewed papers, the all-in/ all-out system was highly advantageous in terms of respiratory disease reduction (Clark et al. 1991; Stark et al.,
1998, Diekman et al., 1999; Maes et al., 1996). High stocking density (Clark et al., 1991; Hurnik et al., 1994; Stark et al., 1998b; 2000) is also a decisive factor in increasing the infection pressure and maintaining respiratory pathogens in the herd.

Biosecurity measures should not be underestimated when control of enzootic pneumonia is concerned. Hege et al. (2002) found that the presence of parking sites for pig transport at a distance of 200m or less from the farm constitutes a potential source for introduction of respiratory diseases. Environmental variables also influence the presence of *M. hyopneumoniae* infections in the farm. Low sunlight levels (Dee et al., 2009), low relative air humidity (Stark et al., 1998a) and low temperature levels, particularly during autumn and winter (Gordon, 1963; Whittlestone, 1976; Goodwin, 1985; Jorsal et al., 1988; Maes et al., 2001) appear to be strongly correlated with the presence of this pathogen in pigs. When considering airborne transmission, not only infected on-farm animals act as infection sources, but also nearby herds with *M. hyopneumoniae* infection and/or respiratory problems. The presence of infected neighbouring farms (Goodwin, 1985; Stark et al., 1992; Hege et al., 2002; Dohoo et al., 2009) and the pig density in the area (Rose et al., 2002; Zhuang et al., 2002) can put herds free of *M. hyopnemoniae* at risk of getting infected.

To effectively control *M. hyopneumoniae* infections in pigs, it is important to eliminate or reduce the effects of each of these risk factors (see chapter 1.1.7).
1.1.5. Symptoms, lesions and diagnostic methods

Clinical signs and lesions

Infection with *M. hyopneumoniae* often appears to have a subclinical course (Clark et al., 1991). However, experimental infection with *M. hyopneumoniae* may lead to coughing, which can be preceded by a slight increase in body temperature. Coughing usually begins approximately two weeks after challenge, reaches its peak after five weeks and declines gradually (Whittlestone et al., 1972; Kobisch et al., 1993). Infections also lead to decreases in average daily weight gain and to increased feed conversion ratio (Maes et al., 1999; Sibila et al., 2009).

Infection leads to consolidation of the lung tissue and catarrhal pneumonia with purple to grey regions of meaty aspect with exudate in the airways (Kobisch et al., 1993). Such consolidation is visible as gross lesions, which are generally observed from 3-12 weeks post infection. The lesions are mainly localized in the apical and cardial lobes, as well as in the anterior part of the diaphragmatic lobes and in the intermediate lobe. Lesions resolve after 12 to 14 weeks with formation of interlobular fissures (Goodwin et al., 1965; Morrison et al., 1985; Blanchard et al., 1992). Complicated infections with *e.g.* secondary bacteria may extend the healing period.

Whittlestone (1972) described the histological changes in the lungs, consisting of neutrophil and lymphocyte infiltration in the lumina, around the airways as well as in the alveoli. As the disease progresses, these cells increase in number in the perivascular, peribronchial and peribronchiolar tissue, forming hyperplastic lymphoid nodules (cuffing) around the airways and leading to alveolar emphysema.

Diagnostic methods
Clinical signs and lung lesions can be used as an indication of enzootic pneumonia. The severity of coughing can be assessed using a respiratory disease score (RDS). The system as described by Halbur et al. (1996) is commonly used and values can range from 0 (no coughing) to 6 (very severe coughing). In addition, post-mortem inspection of lung lesions in pigs at slaughter age (Hurnik et al., 1992; Morris et al., 1995; Sibila et al., 2009) is frequently used to investigate the involvement of *M. hyopneumoniae* in porcine respiratory disease at the herd level. Scoring systems to evaluate respiratory disease based on gross pathological lung lesions have been described by several authors (Hannan et al., 1982; Morrison et al., 1985; Lium and Falk 1991; Hurnik et al., 1993; Ostanello et al., 2007).

In most pig farms with respiratory problems, 20-80% of the pigs at slaughter display pneumonia lesions associated with mycoplasmal infection (Morris et al., 1995; Leneveu et al., 2005; Sibila et al., 2007; Jirawattanapong et al., 2010).

However, assessing clinical symptoms and lung lesion severity are not pathognomonic for diagnosing enzootic pneumonia (Thacker et al., 2001). More accurate diagnostic methods to detect the presence of *M. hyopneumoniae* are necessary to establish a conclusive diagnosis.

Detection of the organism

Bacteriological culture is still the golden standard to identify this organism, but it is seldomly used due to the slow, impractical and laborious process involved (Thacker, 2004; Sibila et al., 2009). Either direct or indirect immunofluorescence testing can be performed on lung tissue, but this cannot be done in life animals (Bradbury, 1998).

Accurate detection of *M. hyopneumoniae* has significantly increased with development of polymerase chain reaction (PCR) assays. The first test to specifically amplify and detect *M.*
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*M. hyopneumoniae* DNA was described by Harasawa et al. (1991). Since then, several PCR tests have been reported (Mattson et al., 1995; Stärk et al., 1998b; Calsamiglia et al., 1999; Caron et al., 2000; Verdin et al., 2000; Kurth et al., 2002; Thacker et al., 2004; Strait et al., 2008). Nested polymerase chain reaction (nPCR) is a useful diagnostic tool developed to enhance the detection threshold of the conventional single-step PCR test (Calsamiglia et al., 2000, Stärk et al., 1998a; Verdin et al., 2000). The advantages of nPCR are its higher sensitivity and specificity. Nested PCR is sufficiently sensitive to detect 1-4 organisms / μl of PCR sample (Kurth et al., 2002; Gebruers et al., 2008). There is however a higher risk for contamination due to repeated pipetting procedures. To avoid false positive results due to contamination, positive and negative controls should be used during DNA extraction and amplification. Lung tissue and BAL fluid appear to be among the most reliable collection sites, especially to detect this pathogen in individual animals (Kurt et al., 2002). Detection of the organism in samples from the nasal cavity appears more variable and is more suitable for detection of *M. hyopneumoniae* at herd level (Otagiri et al., 2005).

Detection of antibodies

Serology is the most common assay used for detection of serum antibodies against *M. hyopneumoniae* at a herd level (Calsamiglia, 1999b; Thacker, 2004). It detects the onset of seroconversion, not the onset of infection. However, not all animals seroconvert at the same time. The detection of serum antibodies after experimental challenge has been reported to range between 2- 6 weeks in nursery pigs (Calsamiglia et al., 1999b). Seroconversion can occur as early as 7- 9 days PI in individual animals (Sheldrake et al., 1990; Sørensen et al., 1997), but it takes 3-6 weeks for all animals to seroconvert in a challenged group (Kobisch et al., 1993; Le Pontier et al., 1994; Leon et al., 2000). In most trials, earlier seroconversion to *M.
"hyopneumoniae" is observed in vaccinated groups compared with non-vaccinated pigs (Sibila et al., 2007). However, there are some limitations when using serological tests, as these can fail due to insufficient sensitivity (Thacker et al., 2006), especially if the infection level in the herd is low. Specificity can be also reduced due to cross-reactions with other species of mycoplasmas such as *M. flocculare* and *M. hyorhinis* (Strasser et al., 1992). Nonetheless, the DAKO® *M. hyo* ELISA is a blocking ELISA based on monoclonal antibodies against the 74 kDa protein of *M. hyopneumoniae* (Feld et al., 1992) with higher sensitivity than previous ELISA tests, preventing cross-reactions with *M. flocculare* and *M. hyorhinis*.

1.1.6. Immune response

Up to date, detailed information about the immune response following *M. hyopneumoniae* infection in pigs is not complete. Following adherence to the pig’s respiratory tract, a series of complex interactions takes place. The host immune system responds with both non-specific and specific defense mechanisms. The non-specific mechanisms include the ‘mucociliary blanket’ acting as the first barrier in the respiratory tract, cytotoxicity of macrophages, increase of T-cell and natural killer cells, as well as the enhancement of the expression of cell receptors that activate the complement cascade (Taylor, 1979; Vicca, 2005a).

The specific response involves systemic and local antibody production, cell-mediated immune stimulation, opsonization and phagocytosis. However, the immune response does not only help in the protection against *M. hyopneumoniae*, but it has also been described to play a role in the exacerbation of lesions following EP (Suter et al., 1985; Turek et al., 1996; Razin et al., 1998; Choi et al., 2006).
After infection with *M. hyopneumoniae*, there is mainly lymphoid cell infiltration around the airways, which is the result of chemoattraction induced by the release of immunogenic proteins and mediators during mycoplasmal infection (Messier et al., 1990; Sarradell et al., 2003). The lymphoid hyperplasia of the bronchus-associated lymphoid tissue (BALT) progressively leads to obliteration of the bronchiole’s lumen and atelectasis of the alveoli (Baskerville et al., 1972; Choi et al., 2006; Redondo et al., 2009). Alveolar macrophages have a very important role in the initiation of this massive lymphoid infiltration, which starts being histologically visible as soon as 7 days after infection (Choi et al., 2006).

An *in vitro* study demonstrated that alveolar macrophages have increased production of IL-1 and IL-6 following *M. hyopneumoniae* infection (Thanawongnuwech et al., 2001). The expression of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-2, IL-4, IL-6, IL8, IL-10, TNF-α and IFN-γ has been reported during infection with this pathogen (Asai et al., 1994; Thanawongnuwech et al., 2003, 2004; Rodriguez et al., 2004; Choi et al., 2006; Lorenzo et al., 2006; Redondo et al., 2009), exerting an effect on Th1 and Th2 response in the respiratory tract of the infected pigs.

Helper T-cells (Th) are an important component in the response to mycoplasma respiratory disease and are the most numerous subset in the lymphoid infiltration. Although less numerous, cytotoxic T-cells are also observed in lungs with *M. hyopneumoniae*- induced lesions (Sarradell et al., 2003). Specifically, IL-1, IL-2, IL-4, IL-6 and TNF-α play a role in lymphoid activation (Baarsch et al., 1995; Murtaugh and Foss, 2002) and exert non specific mitogenic activity (Messier et al., 1990). IL-2 and IL-8 mobilize the immune system in response to infection (Redondo et al., 2009). Upon activation, Th1-cells are responsible for activating and increasing phagocytic and cytotoxic activities of macrophages (Messier et al., 1990; Asai et al., 1994). To a certain extent, the presence of T-cells and overproduction of certain cytokines such as IL-1 and
IFN-γ exacerbate the host mediated tissue damage. Consequently, the pathogenesis of mycoplasmal pneumonia is not only dependent upon direct damage caused by mycoplasma (such as ciliostasis and exfoliation of epithelial cells), but also damage caused by the immune response of the pig itself (Choi et al., 2006). Th1-cells have also been shown to activate B cells to produce opsonizing antibodies such as IgG2 and IgG2b in mice. However, it is the function of Th2-cells to lead B cells to proliferation and antibody production (Chen et al., 2003). As a consequence, a particular increase in the level of IgG and IgA is found in the tracheobronchial secretions, lungs and serum of naturally or experimentally infected pigs (Suter et al., 1985; Messier et al., 1990; Rodriguez et al., 2004; Redondo et al., 2009). IgG participates in opsonization and phagocytosis by alveolar macrophages, whereas IgA seems important in local immunity (Walker et al., 1996; Sarradell et al., 2003). Animals with the lowest M. hyopneumoniae specific antibody levels had the highest macroscopic lung lesion score (Meyns et al., 2006). Also, less pronounced lesions and reduced detection of this microorganism occurred when high amounts of immunoglobulin positive cells were detected in the lungs (Messier et al., 1990).

Results based on detection of Ab should be interpreted carefully as serum Ab measured with commercial ELISAs are generally accepted not to be protective (Haesebrouck et al., 2004). Despite the immune mechanisms of the host, this bacterium is still able to persist in the respiratory tract of the pig. Although lifelong persistence has not (yet) been determined, immunomodulation may enable M. hyopneumoniae to evade or suppress the pig’s defense mechanisms and establish a chronic infection (Razin et al., 1998). An in vivo experiment showed that convalescent carriers of this pathogen can remain infectious for up to 214 days (Pieters et al., 2009). It has been observed that phagocytosis by alveolar macrophages is suppressed during M. hyopneumoniae infection (Caruso and Ross, 1990) and to a greater extent, this
immunosuppression is correlated to a high level of PGE2 in the BAL fluid of infected pigs (Asai et al., 1996). Elevated concentrations of IL-10 and IFN-γ during mycoplasmal infection have also been found to contribute to the inhibition of the phagocytic function of macrophages (Muneta et al., 2008; Redondo et al., 2009).

In addition, it has been suggested that a combination of mechanisms, such as the slow growing rate, the capacity to evade the immune response and the ability of M. hyopneumoniae to colonize cell surfaces (without actual penetration), may prolong the stay of this bacterium within the pig’s respiratory system and hamper its total clearance from the respiratory tract.

Newborn pigs also receive passive immunity by colostrum. Apart from antibodies, colostrum also contains cellular immunity. Colostral lymphocytes are passively transferred from the sows to their offspring (Bandrick et al., 2008). These cells are considered to play a role in the development of the immune response of the piglet. Rautiainen and Wallgren (2001) showed that colostral immunity reduced the severity of lung lesions and the infection rate in young animals (Martelli et al., 2006), but further research is necessary to assess its precise role. Although colostral immunity does not prevent piglet colonization (Thacker et al., 2000; Sibila et al., 2008), a lower seroprevalence at weaning and reduced lung lesions scores at slaughter were observed in pigs originating from sows with a higher concentration of colostral antibodies (Sibila et al., 2008).
1.1.7. Control of *M. hyopneumoniae* infection

Control of enzootic pneumonia can be accomplished through a number of measures that include optimization of herd management and housing conditions (Maes et al., 2008), antimicrobial medication and/or vaccination (Bousquet et al., 1998; Vicca, 2005; Moreau et al., 2004; Martelli et al., 2006; Maes et al., 2008).

1.1.7.1. Herd management and housing conditions

Numerous factors that not only have an impact on the clinical course of enzootic pneumonia, but also on the transmission of *M. hyopneumoniae* have been extensively described by different authors throughout the years (Goodwin, 1985; Thomsen et al., 1992; Hurnik et al., 1994; Stärk 1998b; Stärk, 2000; Maes et al., 2008). Table 2 presents some important management and housing conditions that may enhance the spread *M. hyopneumoniae* infection in a pig herd.

**Optimization of herd management practices**

Improvement of the herd management practices is primordial in the control of *M. hyopneumoniae* infections and should be the first to be accomplished. Instituting management changes that reduce the possibilities of spreading *M. hyopneumoniae* or result in decreased lung damage by other pathogens may lead to considerable improvement in the control of enzootic pneumonia. However, also additional factors different from housing and management conditions, such as strain differences, may determine the infection pattern and clinical course of the disease (Vicca et al., 2002). A review on the influence of environmental and management factors on respiratory disease in pigs, including *M. hyopneumoniae* infections, has been published by Stärk et al. (2000).

*Production system*
All-in, all-out (AIAO) production is probably the most important factor in the control of enzootic pneumonia since it can interrupt the cycle of pathogen transmissions from older to younger pigs. It allows the producer to tailor environmental conditions to a uniform population of pigs and to clean the facilities between groups of pigs. AIAO production also results in better performance and less lung lesions in slaughter pigs (Ice et al., 1997). Mixing or sorting pigs is a source of stress to the animals and it increases the probabilities of disease transmission (Clark et al., 1991). Therefore, an AIAO system in which the same pigs are moved as a group through the different production stages is to be preferred compared to one where pigs are regrouped during transfer from one unit to another.

*Early weaning*

Early weaning (< 3 weeks) can reduce transmission of *M. hyopneumoniae* organisms from the sow to the offspring (Harris et al., 1990; Dee, 1994), but it is not allowed to be applied systematically in the EU. Parity segregation has been used in large production systems as a means to control several diseases in the breeding herd, including *M. hyopneumoniae* infections. Gilts and also their offspring are kept separated from the sows until they reach their second gestation. By that time, they are expected to have acquired the desired immune status and to pose no destabilization risk for the herd anymore.

*Purchase of animals*

Closed pig herds or production systems have a more stable herd immunity compared to herds where (breeding) pigs are purchased. The risk for destabilization of the herd immunity especially increases in case purchasing of animals is performed at a regular basis (Hurnik et al., 1994b), when replacement rates are high, or in case the animals originate from different sources.
(Thomsen et al., 1992). However, the pressure to improve genetics in high performing pig herds forces many pig producers to purchase their breeding stock. In these cases, it is important to evaluate the health status of the herd of origin. Ideally, incoming gilts or any replacements should be of similar or higher health status than the recipient herd to prevent them from introducing infections not present in the recipient herd. A quarantine or adaptation period of at least 4 weeks should be respected.

*Animal stocking density*

Decreasing animal density during the different production stages has been shown to reduce the level of respiratory disease (Flesja and Solberg, 1981; Hurnik et al., 1994a). Crowding may lead to increased transmission of pathogens, and to stress reactions, making the pigs more susceptible to infectious diseases. Too low stocking densities are financially not justified. Therefore, it is important to find a reasonable compromise between stocking densities that are appropriate for the health of the pigs and those that maximize the returns on the building’s cost. In general, the stocking density for finishing pigs on fully slatted floors should equal or exceed 0.7 m² per pig (Lebret et al., 2006).

*Prevention of other diseases*

Appropriate parasite control measures are necessary to avoid any lung damage caused by migrating *Ascaris suum* larvae (Jolie et al., 1998). Lung damage may also arise from infections with other respiratory pathogens including bacteria and viruses. PRRSV, influenza viruses, PCV-2 and PRCV, in combination with *M. hyopneumoniae*, are considered to play an important role in the development of PRDC (Thacker et al., 2002; Bochev, 2008). The impact of these
infections can be reduced by means of medication, vaccination, improvement of housing conditions and implementation of biosecurity measures.

**Biosecurity measures**

Spread of *M. hyopneumoniae* within sites and within herds can be controlled by applying basic biosecurity measures. Some of these measures (concerning purchasing policy, prevention of other diseases, etc) have been already mentioned. But these measures should also encompass appropriate hygienic conditions for rearing pigs, surveillance, disease outbreak control and prophylactic measures (Amass et al., 1999). Also personnel, vehicles for transport, feed, water, bedding and equipment should be separated by production stages and should not come in contact with animals of different ages (Hege et al., 2002; Roman et al., 2006; Stankovic et al., 2010). A biosecurity plan should take into account epidemiological situations, and potential threats to the herd health and production, as well as possible solutions.

**Improvement of housing conditions**

Housing and/or environmental changes that optimize the climate of the pigs' environment are important in the control of *M. hyopneumoniae* infections. Special attention should be paid to the temperature set points, fan staging, air inlet and curtain settings, sensor placement, heater capacity, drafts and building maintenance (Heinonen et al., 2001; Maes et al., 2008; Mul et al., 2010). However, making environmental changes for improving the climate in inappropriate or old barns frequently entail extensive remodeling, and therefore, they may be difficult and expensive to institute and maintain.
### Table 2: Risk factors influencing infection with *M. hyopneumoniae* in a pig herd

<table>
<thead>
<tr>
<th><strong>Herd Factors</strong></th>
<th><strong>Author(s)</strong></th>
</tr>
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<tbody>
<tr>
<td>Increasing herd size</td>
<td>Goodwin, 1985; Stark et al., 1992; Stärk et al., 1998b; Maes et al., 2000; Stärk, 2000</td>
</tr>
<tr>
<td>Herd type: growing-finishing farm</td>
<td>Clark et al., 1991; Hofer, 1993; Hege et al., 2002; Maes et al., 2008</td>
</tr>
<tr>
<td><strong>Housing and Management</strong></td>
<td></td>
</tr>
<tr>
<td>Increasing stocking density in the finishing unit</td>
<td>Done et al., 1991; Hurnik et al., 1994; Stärk et al., 1992; Stärk et al., 1998b; Clark et al., 1991; Stärk et al., 1998b; Diekman et al., 1999</td>
</tr>
<tr>
<td>Continuous production system</td>
<td>Thomsen et al., 1992; Stärk, 2000; Hege et al., 2002</td>
</tr>
<tr>
<td>Multi-source purchase</td>
<td>Maes et al., 2000</td>
</tr>
<tr>
<td>Purchase of gilts</td>
<td>Hege et al., 2002</td>
</tr>
<tr>
<td>Parking site for pigs transport vehicles closer than 200m to the farm</td>
<td></td>
</tr>
<tr>
<td><strong>Weather variables</strong></td>
<td></td>
</tr>
<tr>
<td>Low temperature (autumn and winter)</td>
<td>Gordon, 1963; Whittlestone, 1976; Goodwin, 1985; Jorsal et al., 1988; Thomsen et al., 1992; Maes et al., 2001 Dee et al., 2009</td>
</tr>
<tr>
<td>Low sunlight intensity</td>
<td>Dee et al., 2009</td>
</tr>
<tr>
<td>Low air humidity</td>
<td>Stark et al., 1998a</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td>Neighboring farms infected with <em>M. hyopneumoniae</em></td>
<td>Goodwin, 1985; Stärk et al., 1992; Thomson et al., 1992; Hege et al., 2002; Rose et al., 2002; Zhuang et al., 2002; Dohoo et al., 2009</td>
</tr>
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</table>

### 1.1.7.2. Antimicrobial medication

The lack of a cell wall makes this organism insensitive to the group of antibiotics belonging to β-lactam group, such as penicillins and celophalosporins (Braun, 1970; Wu et al., 1997). Macrolides, tetracyclines and lincosamides include the most commonly used antibiotics in the control of respiratory diseases in pigs (Timmerman et al., 2006) and have been described to be also effective against mycoplasma. The use of antibiotics in feed and water is common practice, which efficacy is often related to the reduction of clinical symptoms, lung lesions, improvement
of performance parameters and prevention of secondary infections (Stipkovitz et al., 2001; Vicca et al., 2005; Thacker et al., 2006). Although antibiotic treatments are usually able to control the disease (Bargen et al., 2004; McKelvie et al., 2005), persistence is observed under field conditions (Ross, 1999) and experimental infections (Kobisch et al., 1996; Hannan et al., 1997; Le Carreau et al., 2006).

During critical stages in production, strategic medication can be used. However, the preventive or strategic use of antimicrobials should be discouraged since it increases the risk of antimicrobial resistance and in case of inappropriate use, may also lead to antimicrobial residues in the pigs carcasses at the moment of slaughter (Maes et al., 2008). Acquired resistance of *M. hyopneumoniae* under experimental conditions has been documented to tetracyclines and some macrolides (Inamoto et al., 1994; Hannan et al., 1997), and lately also to lincosamides and fluoroquinolones (Vicca et al., 2004; 2007). The advantages of using antimicrobials compared to vaccination include flexibility in their usage and easy administration with feed or water, which makes them not so labour intensive. Despite the success of medication programmes in the control of *M. hyopneumoniae* infections described by various studies (Jouglar et al., 1993; Walter et al., 2000), often only partial prevention is achieved and sometimes results are inconsistent since outbreaks may reappear after ceasing treatment (Wallgren et al., 1993; Thacker et al., 2006).

**1.1.7.3. Vaccination against *Mycoplasma hyopneumoniae***

Vaccination with inactivated, adjuvanted whole-cell bacterins (alone or in combination with antibiotics) is frequently used worldwide to control *M. hyopneumoniae* infections (Erlandson et al., 2002).
1.1.7.3.1. **Vaccination strategies**

Numerous trials have proven the efficacy of vaccination against *M. hyopneumoniae* to reduce clinical disease and *Mycoplasma* lung lesions and to increase performance parameters of the pigs (Jensen et al., 2002; Maes et al., 2003; 2008).

It has been also suggested that vaccination decreases the infection level in the herd (Sibila et al., 2007) and although vaccination does not offer complete protection nor prevents pigs from becoming infected (Mateusen et al., 2001; 2002; Meyns et al., 2004), vaccination may reduce the number of *Mycoplasma* organisms in the respiratory tract of the pig (Meyns et al., 2006). Despite the fact that vaccination against *M. hyopneumoniae* is widely practiced, many aspects about its effect on transmission of the pathogen, effect on different strains and on the immunological response remain unclear and need to be further investigated.

Different vaccination strategies have been adopted depending on the needs of a particular herd and on variables such as the production system, type of herd, infection pattern and preferences of the pig producer. The decision on whether to vaccinate or not depends on the economic feasibility in the farm and the profits that vaccination will bring in relation to the economic losses caused by the pig’s decreased performance. In other words, vaccination not only has to be efficacious, but also cost-effective (Maes et al., 2003; 2008).

Generally, in Europe, piglets are vaccinated at or before weaning. Vaccination is mostly effective if active immunity is established before infection, making this a critical factor in the implementation of vaccination strategies. Initially, the most frequently used vaccines were double-shot bacterins administered intramuscularly in the first week of life, and repeated 2-3
weeks later. Notwithstanding their current availability, single-shot *M. hyopneumoniae* vaccines have become more popular due to their reduced labor costs and less stress inflicted to the piglets (Baccaro et al., 2006). There are still some debates as for which vaccination program is more appropriate. For example, in the US, single dose vaccines are recommended for herds with low infection pressure and where an all-in/all-out production is practiced (Yeske, 2001). However, several studies have shown that both single and double shot vaccination programs have similar protective effects (Morris et al., 2001; Roof et al., 2001; Haesebrouck et al., 2004). Some authors suggest that the efficacy of vaccination in young piglets may depend on the level of maternally derived antibodies at the time of implementing vaccination (grosse Beilage et al., 2005; Martelli et al., 2006; Bandrick et al., 2008). The role of maternal cellular immunity and antibody mediated immunity in the protection of newborn piglets is not entirely clear and the potential interference with active immunity needs to be better understood. Despite the discrepancies in literature and ongoing debate on whether maternal antibodies interfere or not with vaccination efficacy, almost all pigs in Europe are vaccinated in presence of maternal antibodies with very good results (Martelli et al., 2006; Maes et al., 2008).

Apart from the active immunization of piglets, also vaccination of gestating sows can be done. However, the question of whether to vaccinate sows before farrowing or not, remains unanswered. In practice, sow vaccination is rarely practiced and the main strategy to control *M. hyopneumoniae* infection is based on piglet vaccination. (Haesebrouck, 2004). The main purpose of sow vaccination is to decrease the spread of *M. hyopneumoniae* from mother to offspring and to ensure protection of newborn piglets through the passage of maternally derived antibodies. Some studies have shown that sow vaccination before farrowing can lower infection pressure and have an effect on vertical transmission, resulting in lower piglet colonization by *M.*
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*mycoplasma hyopneumoniae* (Ruiz et al., 2003). However, colostral immunity does not prevent colonization of the piglets with *M. hyopneumoniae*.

Sow vaccination protocols are especially recommended as a biosecurity measure in herds purchasing gilts from *M. hyopneumoniae* free farms. Another reason of gilt vaccination at the moment of introduction in the breeding population is to prevent them from destabilizing the infection level in the herd or as a response to an outbreak of enzootic pneumonia in the farm (Bargen et al., 2004).

1.1.7.3.2. **Effect of vaccination on transmission of *M. hyopneumoniae***

Meyns et al. (2004) estimated that one infected animal will infect on average one susceptible penmate during the nursery period. These results emphasize the importance of the implementation of control strategies from an early age. In another transmission study, the same authors (Meyns et al., 2006) showed that vaccination was efficacious to reduce clinical symptoms and lung lesions, but that the transmission of *M. hyopneumoniae* was only numerically lower in the vaccinated group (Rn= 2.38), compared to the non-vaccinated group (Rn= 3.51). This implies that vaccination alone will not lead to elimination of this pathogen in a herd. Whether these results are also valid under field conditions is not known.

1.1.7.3.3. **Development of new vaccines against *M. hyopneumoniae***

The first vaccines tested against *M. hyopneumoniae* were inactivated whole cell bacterins (Ross et al., 1984; Kobisch et al., 1987) and adjuvanted bacterins are still the ones that are currently
used commercially (Okada et al., 1999). Constant effort is being directed towards the investigation of other *M. hyopneumoniae* strains, protective antigens, and routes of administration that may offer a better protection against *M. hyopneumoniae* infections.

Apart from the conventional intramuscular administration, other alternative routes of immunization have been described for pigs. These include intraperitoneal immunization (Sheldrake et al., 1991; Ciprian et al., 1994), vaccines in aerosol (Murphy et al., 1993); oral microcapsules (Lin et al., 2003); intradermal application (Jones et al., 2005) and intrapulmonic immunization (Feng et al., 2010). Although most of these vaccines have shown to be efficacious in the reduction of lung lesions and clinical symptoms under experimental and field conditions, some of them have not been successful and have not demonstrated sufficient economic improvement in performance parameters. More studies need to be conducted in order to validate these vaccines under experimental and practical circumstances.

In addition, the route of vaccination can influence the type of immune response triggered in an animal. For instance, the same subunit vaccine against *M. hyopneumoniae* (rLTBR1), composed of R1 repeat region of P97 adhesin, induced a better Th2 response when administered intramuscularly and induced a more accentuated Th1 response when administered intranasally in mice (Conceição et al., 2006). Furthermore, some of these experimental vaccines require a more labour intensive way of administration and are more expensive, which not always makes them commercially justifiable (Jakab et al., 1995).

Aerosol and oral vaccine delivery have received special attention as alternative routes, since these would allow mass vaccination, significantly reduce labour cost, increase mucosal immunity in the respiratory tract (Lin et al., 2003) and decrease stress caused by individual handling during
vaccination. Unfortunately, so far, they are less protective than the intramuscular vaccination (Murphy et al., 1993).

Apart from the antigen in the vaccine, also the adjuvant plays a role in protection. Most commercial formulations against *M. hyopneumoniae* contain oil-based adjuvants to prolong the release of the antigen and stimulate the immune response of the host (Groth et al., 2001). Nevertheless, the advantages of different adjuvants are still under debate. More studies are needed to investigate the effect of different formulations.

Another concern in the search for new vaccines is that protective species-specific antigens against *M. hyopneumoniae* still remain largely undefined and most of the formulations encompassing single antigens tend to offer only partial protection. Recently, efforts are being made on a molecular basis to map more epitopes that could potentially serve as components in future vaccines (Talaat et al., 2005; Chen et al., 2008). The expression library immunization (ELI), for instance, is a novel technique to screen genomes in order to identify potential vaccine candidates (Talaat et al., 2005). Given its small genome, ELI was first used in *M. pneumoniae*. A small genome is a characteristic also shared by *M. hyopneumoniae*. Up to date, three different strains have been sequenced, namely J, 232 and 7448 (Minion et al., 2004; Vasconcelos et al., 2005), making this pathogen a particularly suitable candidate for the ELI technique. The utilization of ELI in a pig model and the development of improved vectors for screening recombinant proteins were first reported by Moore et al. (2001). A recent study describes the cloning, expression and purification of thirty new recombinant *M. hyopneumoniae* proteins using *E. coli* as expression vector (Simionatto et al., 2010).

A combination of both novel and conventional methodologies has led to the identification of a few specific antigens of *M. hyopneumoniae*, among which figures the lipoprotein P65 (Kim et
al., 1990), cytosolic P36 protein, a L-lactate dehydrogenase (Strasser et al., 1991), surface lipoproteins P46 (Futo et al., 1995), P97 protein (Zhang et al., 1995), P97R1, ribonucleotide reductase – NrdF (Fagan et al., 1996) and lipoproteins Mhp378 and Mhp651 (Meens et al., 2006). Although most of these molecules have a great potential as vaccine candidates, P97, P97R1 and NrdF have been predominantly evaluated in various experimental studies that include animal models (King et al., 1997; Fagan et al., 1997; Chen et al., 2001; 2003; 2006).

P97 plays an important role in the adherence of *M. hyopneumoniae* to the cilia (Zhang et al., 1995; Minion et al., 2000) and ever since it was identified, it has been the most studied and best defined potential protective antigen against *M. hyopneumoniae*. It has been used in experimental subunit vaccines for mice and pig infection models (Chen et al., 2001; Conceicao et al., 2006). The first use of P97 as a subunit vaccine provided only minimal, but not significative protection against lung lesion (King et al., 1997), but subsequent studies have reported better protection and higher antibody titers than with conventional vaccines (Chen et al., 2001). In another trial, an attenuated *Erysipelothrix rhusiopathiae* YS-19 vaccine expressing the C-terminal portion of the P97 adhesin was intranasally administered to pigs (Shimoji et al., 2003). It significantly reduced the severity of pneumonic lung lesions, but failed to produce P97-specific serum antibodies. Ogawa et al. (2009) reported that oral immunization with the same vaccine did not provide successful results. The failure via the oral route could have been due to over-attenuation of the strain. As a consequence, a live vaccine was developed against *M. hyopneumoniae*, using the *E. rhusiopathiae* strain as a vector expressing the C-terminal domain of the P97 adhesin (Ogawa et al., 2009). This time, the oral vaccine successfully induced protection against mycoplasmal pneumonia, significantly reducing the severity of lung lesions.
P97R1 (also called R1) is a repeat of P97 that contains both cilium and antibody binding sites (Hsu and Minion, 1998; Hsu et al., 1997) and has an immunogenic effect independently from other P97 regions (Minion et al., 2000). Generating anti-P97R1 mucosal immune response might play a role in the prevention of colonization of the pig’s respiratory tract by *M. hyopneumoniae* (Sheldrake et al., 1993; Thacker et al., 2000). In an experiment performed by Chen et al. (2006), mice immunized with the vaccine carrier *Salmonella* Typhimurium aroA CS332 generated a cell-mediated response and serum IgG, but not mucosal IgA against P97R1, questioning the suitability of the *S.* Typhimurium antigen-carrier as a vector encoding P97R1.

On the other hand, NrdF shows homology to the prokaryotic R2 subunit of ribonucleotide reductase (Fagan et al., 1996). In an experiment, pigs immunized with a 11kDa protein containing the C-terminal of NrdF, had significantly lower lung lesions after being infected with *M. hyopneumoniae*, but there was no difference in weight gain in comparison with the control animals. One year later, the same authors immunized mice orally with *Salmonella* Typhimurium aroA strain SL3261 expressing NrdF fragment, producing significant specific IgA anti-NrdF in the lungs, but there were no significant levels of IgG, IgM or IgA in the serum (Fagan et al., 1997). A similar trial was performed in pigs, immunizing them orally with the same attenuated *S.* Typhimurium aroA expressing a recombinant antigen of *M. hyopneumoniae* (NrdF) (Fagan et al., 2001), demonstrating this time reduced lung lesions and higher daily weight gain compared to the non vaccinated animals. An orally delivered vaccine in mice evoked NrdF-specific IFN-γ and cellular mediated immunity by using a eukaryotic expression vector (Chen et al., 2006).
Despite the immunizing properties of all of these proteins and the reduced severity of lung lesions, none of them is currently able to offer total protection or a similar protection as the commercial vaccines. Multivalent formulations could be another alternative in offering a better protection against *M. hyopneumoniae* (Chen et al., 2008). Inoculation with plasmid DNA, encoding immunogenic proteins for a specific pathogen, has emerged as a novel approach for developing new generation vaccines (Dhama et al., 2008). Cocktail DNA vaccines elicit both cell mediated and humoral immunity. They can be administered in different ways (intramuscular, subcutaneous or oral), and they can also contain recombinant plasmids with multiple potential antigens. A multivalent DNA vaccine with different immunogenic proteins against *M. hyopneumoniae* (P36, P46, NrdF, and P97 or P97R1) has been tested in mice (Chen et al., 2008). The major findings were that intramuscular immunization induced a strong Th1 immune response, whereas the humoral response was elicited only by P46. Subcutaneous immunization, on the other hand, triggered both humoral and Th1 response. In addition, P97 was not recognized by serum antibodies in mice immunized with a commercial bacterin, which might be an indication of the lack of expression of this antigen in inactivated whole-cell vaccines.

The major limitations faced nowadays with DNA vaccines is that while they enable a long lasting protection effect in small experimental animals, less potent immune responses are achieved in larger animals. Moreover, plasmid DNA is easily degradable *in vivo*, requiring better delivery systems for the vaccine to remain efficacious (Dhama et al., 2008).

Although the novel methodologies and new antigens may show promising effects for the control of *M. hyopneumoniae* infections, many hurdles are still to be taken to achieve a better control than with the current commercially available vaccines.
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CHAPTER 2.
AIMS OF THE STUDY
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*M. hyopneumoniae* is the primary agent of enzootic pneumonia, a chronic respiratory disease in pigs that occurs worldwide and causes major economic losses. The clinical course of infections may vary significantly between pig herds depending on herd management strategies and environmental conditions. However, also the virulence of the *M. hyopneumoniae* strains may determine the clinical outcome, which renders control of this pathogen a difficult task. Control measures include the optimization of management and housing conditions, the use of strategic medication and/or vaccination. The current control measures are beneficial from an economic point of view, but they do not entail a sustainable solution for the disease.

There is limited information on the occurrence and risk factors involved in early infection and there are still many questions regarding the effect of vaccination on transmission of this pathogen. The limited studies available have mainly focused on experimental conditions and do not necessarily reflect the situation in the field. In addition, information on differences between infection course caused by low and highly virulent field strains and the effect of vaccination on infection with different *M. hyopneumoniae* strains is very scarce. Answers to these questions are necessary for a better understanding of the pathogenicity of this microorganism, and to improve the control of enzootic pneumonia.

The general aim of this study was to assess the importance of early infection and transmission of *M. hyopneumoniae* under field conditions and to investigate protection against different *M. hyopneumoniae* strains.

The specific objectives were:
1. To estimate the detection rate of *M. hyopneumoniae* infections in piglets at 3 weeks of age in different European countries by analysing nasal swabs using nPCR,

2. To quantify the transmission of *M. hyopneumoniae* in nursery pigs under field conditions, and to assess the effect of vaccination,

3. To assess the efficacy of a commercial vaccine against infection with *M. hyopneumoniae* strains of low and high virulence,

4. To investigate the infection pattern and lung lesion development in pigs experimentally infected with low and highly virulent *M. hyopneumoniae* strains,

5. To investigate the effect of infection with low virulent *M. hyopneumoniae* strains on a subsequent challenge infection with a highly virulent strain.
CHAPTER 3.

EXPERIMENTAL STUDIES
Early infection and transmission of *Mycoplasma hyopneumoniae* under field conditions
3.1 Early *Mycoplasma hyopneumoniae* infections in suckling pigs in herds with respiratory problems in Europe: detection rate and risk factors

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Abstract

The present study aimed to estimate the detection rate of *M. hyopneumoniae* in 3-week-old pigs in different European countries and to identify possible risk factors. Nasal swabs from suckling pigs in 52 farms were collected for analysis using nested PCR. Potential risk factors for respiratory disease were analysed with a multivariable logistic regression model. The average percentage of positive piglets was 10.7% (95% confidence interval, CI 7.4-14.2); at least one pig tested positive in 68% of herds. In 32% of the herds, more than 10% of piglets tested positive. Herds that vaccinated sows against swine influenza virus (SIV) had a significantly higher risk of a piglet being positive for *M. hyopneumoniae* (OR 3.12; 95% CI 1.43-6.83). The higher risk in case of SIV vaccination is difficult to explain, but it may be due to the fact that pig herds with respiratory symptoms are more likely to be vaccinated against SIV, overlooking the possible influence of other respiratory pathogens such as *M. hyopneumoniae*. The present findings show that *M. hyopneumoniae* is widespread in 3-week-old piglets across different European countries.
1. Introduction

Infections with *Mycoplasma hyopneumoniae* occur worldwide and cause major economic losses to the pig industry. Control of *M. hyopneumoniae* can be accomplished by optimising management and housing practices, by antimicrobial medication and by vaccination. In many European countries, more than half of the pig herds practice vaccination to control the infection (Maes et al., 2008). Different vaccination protocols are used, but in most instances the piglets are vaccinated once or twice in the farrowing unit or at weaning.

The spread of *M. hyopneumoniae* occurs by either vertical transmission from the sow to the offspring (Sibila et al., 2007) or by horizontal transmission between pigs (Meyns et al., 2004; Fano et al., 2007). Sows transmit *M. hyopneumoniae* to the offspring mainly by direct nose-to-nose contact, as there is no transmission via the intra-uterine route, nor by colostrum or milk. The importance of the sow for infection of the piglets is not clear and has been investigated only in a few studies including a limited number of pig herds. Goodwin et al. (1965) reported that younger sows are more likely to transmit *M. hyopneumoniae* to their piglets than older sows. Older sows, especially those in endemically infected herds, have a lower probability of harbouring *M. hyopneumoniae* in their respiratory tract (Clark et al., 1991). Calsamiglia and Pijoan (2000) found a higher percentage of young sows infected with *M. hyopneumoniae* in their upper respiratory tract, but the microorganism was also demonstrated in nasal samples of older sows (3rd to 7th parity).

Studies using nested PCR (nPCR) on nasal swabs from piglets before weaning in a limited number of herds have been performed. Calsamiglia and Pijoan (2000) tested animals in a three-
site system and found 7.7-9.6% positive pigs at 17 days of age. Ruiz et al. (2003) found 5.5-13.2% of piglets positive on a breeding farm at 19 days of age. Sibila et al. (2007) performed nasal sampling in piglets from 1 and 3 weeks of age; the percentage of positive piglets ranged between 0 and 6.4%. Much higher infection rates were obtained by Fano et al. (2007). They reported a prevalence of 2.5-51.8% in piglets at one day before weaning (17 days of age), in 11 batches of a multi-site production farm. Although the presence of DNA in nasal swabs is not a 100% guarantee for infection, it is certainly an indication that these animals were exposed to the organism.

Sibila et al. (2007) performed nPCR on bronchial and tonsillar swabs of 37 pigs at 3 weeks of age and they showed the presence of mycoplasmal DNA in the tonsils of one pig and in the bronchial swab of two other pigs. Infection of piglets during the suckling period is particularly important, since piglets are transferred to other pens and are usually regrouped with other piglets at weaning. In this way, infected piglets may easily transmit the infection to other non-infected pigs during nursery.

To implement appropriate control measures and to further optimise the vaccination strategies, it is important to have a precise notion of how widespread this agent is in young pigs from different pig herds in different countries. Knowledge on early infection is particularly important for the European pig industry, as early vaccination is widely practised (Maes et al., 2003) and a major part of the industry are farrow-to-finish pig herds. Apart from assessing the infection rate, it is also important to identify possible herd factors that may predispose to early infections in pigs. The aim of the present study was to estimate the detection rate of *M. hyopneumoniae* infections in piglets at 3 weeks of age in different European countries by analysing nasal swabs.
using nPCR. In addition, possible risk factors associated with *M. hyopneumoniae* infection in young piglets were investigated.
2. Materials and methods

Study herds

The study was conducted from May 2008 to March 2009 in nine European countries (Belgium, Denmark, France, Germany, Hungary, Italy, Poland, Spain and The Netherlands). The target population consisted of 52 pig herds. In each country, 6 single-site farrow-to-finish or sow herds were included, except for Germany, where 4 herds were included. The herds had to comply with specific selection criteria, such as a minimum herd size of 100 sows, presence of clinical respiratory problems related to *M. hyopneumoniae* (coughing in grower-finishing pigs) and no use of antimicrobials active against *M. hyopneumoniae* in piglets less than 3 weeks of age.

Nasal swabs

To obtain with 95% certainty a detection rate of at least 10% in the selected countries, a minimum of 30 piglets had to be sampled per herd. Hence, 30 nasal swabs were collected in each herd from piglets of 21 ± 3 days of age. The piglets within each herd were selected randomly from as many different sows as possible. In case there were less than 30 mother sows, two piglets per sow were selected.

Nasal swabs were obtained by deeply swabbing the nasal mucosa of one nostril using a cotton-tipped swab. The samples from each country were stored at -70 °C.
Analysis of nasal samples

The material collected in each nasal swab was suspended in 200 µL phosphate buffered saline (PBS) and DNA was extracted using the Qiagen, DNAeasy Blood & Tissue Kit. To detect *M. hyopneumoniae* DNA, a nPCR protocol was used (Villarreal et al., 2009). The nested PCR reactions were performed using Taq polymerase. In the first step, a final volume of 20 µL contained 2 µL 10x PCR buffer, 2 µL 3 mM MgCl₂, 0.36 µL 170 µM dNTPs, 12.5 µL of high performance liquid chromatography water (HPLC-H₂O), 0.1 µL 0.03 U/µL Taq polymerase (Gibco Invitrogen), 0.5 µL each primer MHP950-1L (5’-AggAACACCATgCgATTTTTA-3’) and MHP950-1R (5’-ATAAAAATggCATTTCCCTTCA-3’) and 2 µL 10-fold-diluted DNA solution.

In the second step, a final volume of 15 µL contained 1.5 µL 10x PCR buffer, 0.9 µL 3 mM MgCl₂, 0.27 µL 170 µM dNTPs, 10 µL HPLC-H₂O, 0.08 µL 0.03 U/µL Taq polymerase, 0.5 µL primers MHP950-2L (5’-CCCTTTgTCTCAATTGTgCAA-3’) and MHP950-2R (5’-gCCgATTCTAgTACCCTAATCC-3’) and 1.5 µL 10-fold-diluted DNA solution.

The following PCR programme was used for both steps: initial denaturation at 94 °C for 2 min; denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s and extension at 72 °C for 1 min; 35 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min; final extension at 72 °C for 5 min, followed by cooling to 10 °C. Nested PCR products were analysed by electrophoresis on 1.5% agarose gels in Tris-Borate-EDTA (TBE) buffer and visualised under ultraviolet illumination after staining with GelRed (Biotium). Positive and negative controls were used for both DNA extraction and amplification.
Herd data collection

A questionnaire related to respiratory disease and herd management practices was completed during a face-to-face interview with the farmer and when inspecting the sheds for each sampled herd. Thirty eight questions were included in the analysis. To ensure standardisation of the answers, most of these questions were closed (28) or semi-closed (10). The questionnaire was pretested in three pig herds before the start of the study. The questions were divided in six categories such as general herd information, sow population, purchase policy, housing conditions, feeding management and health control strategies. For logistical and language reasons, the completion of the questionnaires and the sampling were performed by the herd veterinarians in each country.

Statistical analyses

The detection rate in each herd was based on the number of positive nasal swabs detected by nPCR. In order to determine the risk factors associated with the detection rate of \textit{M. hyopneumoniae} at herd level, the PROC GLIMMIX procedure (SAS 9.2, 2008) was used to fit a generalised mixed model for the presence of \textit{M. hyopneumoniae} with country and herd nested in country as random effects and binomially distributed error term. The predictor variables with $P \leq 0.10$ in the univariate models were subsequently included in the multivariable analysis. A backward likelihood ratio selection criterion was used for the multivariable regression model in order to choose the variables that were significantly associated with the outcome variable ($P \leq 0.05$) at the multivariable level. At each step, a variable with the highest $P$ value (and $>0.05$)
was excluded from the model until no additional variables could be removed. One-way biologically plausible interactions between the independent variables were tested in the final model. The goodness-of-fit was evaluated by the ratio of the $\chi^2$ statistic and the corresponding degrees of freedom with a value close to 1. Results were summarised using odd ratios with their 95% confidence intervals.
3. Results

In total, 52 herds and 1,555 piglets were sampled for presence of *M. hyopneumoniae*. Of all herds included in the study, 28.6% were farrow-to-finish herds, 26.5% and 24.5% were sow herds from which the piglets were moved to another farm after weaning or at 20-25 kg, respectively, and the remainder of the herds (20.4%) raised a part of the fattening pigs on the same site and partly moved pigs after weaning or at 10 weeks of age. The sows from the sampled pigs had following parity distribution: 1st to 2nd parity: 39%; 3rd to 4th parity: 30%; 5th to 7th parity: 27%; and >7th parity: 4%. Twenty eight percent of the herds vaccinated sows against *M. hyopneumoniae* before their first gestation.

The total average percentage of positive pigs within the herds was 10.7% (95% CI 7.4-14.2). In herds vaccinating against *M. hyopneumoniae* 8.4% (95% CI 7.6-9.1) were infected, whereas only 2.6% (95% CI 1.8-3.4) were infected in the herds not vaccinating against *M. hyopneumoniae*. Twenty eight percent of the herds practised sow vaccination against *M. hyopneumoniae* before their first gestation, accounting for 3.1% of the *M. hyopneumoniae* positive piglets. No significant difference was found between these and the herds not practicing sow vaccination (P>0.05).

The minimum within-herd detection rate was 0% and the maximum within-herd detection rate was 37% (Fig. 1). The percentage of herds with at least one positive tested pig was 68% (95% CI 51.7-83.4).
Fig 1. Distribution of herds according to within-herd detection rate of *M. hyopneumoniae* in 3-week old piglets. Fifty-two pig herds from different European countries were included; 30 piglets per herd were sampled.

The number of positive herds and positive animals in the different countries are presented in Table 1. France, The Netherlands, Italy and Germany were the countries with the highest number of herds positive for *M. hyopneumoniae*. In France, 100% of the herds were positive. The highest within-herd detection rates were found in Italy, France and Germany.
Table 1: Detection rate of *M. hyopneumoniae* in nasal swabs of 3-week old pigs in different EU countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>Number of positive herds/Number of herds sampled</th>
<th>Number (%) of positive piglets&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Minimum-maximum % of positive pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>4/6</td>
<td>6 (3.3)</td>
<td>0.0-10.0</td>
</tr>
<tr>
<td>Denmark</td>
<td>3/6</td>
<td>3 (1.7)</td>
<td>0.0-3.30</td>
</tr>
<tr>
<td>France</td>
<td>6/6</td>
<td>38 (21.1)</td>
<td>10.0-30.0</td>
</tr>
<tr>
<td>Germany&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3/4</td>
<td>23 (18.7)</td>
<td>0.0-26.7</td>
</tr>
<tr>
<td>Hungary</td>
<td>3/6</td>
<td>18 (10.0)</td>
<td>0.0-30.0</td>
</tr>
<tr>
<td>Italy</td>
<td>5/6</td>
<td>40 (22.1)</td>
<td>0.0-36.7</td>
</tr>
<tr>
<td>Poland</td>
<td>4/6</td>
<td>17 (9.7)</td>
<td>0.0-26.7</td>
</tr>
<tr>
<td>Spain</td>
<td>2/6</td>
<td>9 (5.0)</td>
<td>0.0-20.0</td>
</tr>
<tr>
<td>The Netherlands</td>
<td>5/6</td>
<td>14 (7.7)</td>
<td>0.0-16.1</td>
</tr>
<tr>
<td>Total (%)</td>
<td>35/52 (67.3)</td>
<td>168/1555 (10.7)</td>
<td>0.0-36.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fifty-two herds were sampled; 30 piglets were sampled in each herd: 6 herds per country, 30 pigs per herd = 180 pigs per country (only 4 herds or 120 pigs in Germany). Only 25 samples were taken from one herd in Poland.

<sup>b</sup> Only 4 herds were sampled.
Three of 38 variables were significant in the univariate analysis (P ≤0.10), namely sow vaccination against swine influenza (SIV), sow vaccination against atrophic rhinitis and the presence of a slaughterhouse within a distance of 5 km from the herd (Table 2). In total, 27% and 28% of herds vaccinated their sows against SIV and atrophic rhinitis, respectively. In 10% of the herds, a slaughterhouse was located within 5 km of the herd.
Table 2: Potential risk factors \((P <0.10)\) associated at herd level with the detection rate of \(M.\) hyopneumoniae in piglets of 3 weeks of age after univariate analysis.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Number of herds</th>
<th>Number (%) of herds per level</th>
<th>Mean % of positive herds per level</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sow vaccination against swine influenza</td>
<td>52</td>
<td>38 (73.1)</td>
<td>5.0</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 (26.9)</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td>Sow vaccination against atrophic rhinitis</td>
<td>52</td>
<td>37 (71.2)</td>
<td>5.4</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 (28.2)</td>
<td>13.4</td>
<td></td>
</tr>
<tr>
<td>Presence of slaughterhouse within 5 km</td>
<td>49</td>
<td>44 (89.8)</td>
<td>6.6</td>
<td>0.034</td>
</tr>
<tr>
<td>from pig herd</td>
<td></td>
<td>5 (10.2)</td>
<td>21.6</td>
<td></td>
</tr>
</tbody>
</table>

These three variables were subsequently used in the multivariable model. As presented in table 3, only sow vaccination against SIV was significantly \((P <0.05)\) and positively associated with the prevalence of \(M.\) hyopneumoniae in the piglets \((OR 3.12; 95\% \text{ CI} 1.43-6.83)\) when adjusted for the other covariates. Vaccination against atrophic rhinitis was not significantly associated with the prevalence of \(M.\) hyopneumoniae in piglets, but the factor was retained in the final model because the \(P\) value was borderline not significant \((P = 0.052)\). The goodness of fit statistic was 0.95, close to and not differing significantly from 1, demonstrating that this model describes the data well.
Table 3: Risk factors ($P<0.05$) for being *M. hyopneumoniae* positive in 3-week-old piglets in nine European countries in the final multivariable logistic regression model$^a$

<table>
<thead>
<tr>
<th>Variable</th>
<th>Level</th>
<th>β-coefficient</th>
<th>Odds ratio (95% CI)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sow vaccination against swine influenza</td>
<td>No</td>
<td></td>
<td>1</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1.14</td>
<td>3.12 (1.43-6.83)</td>
<td></td>
</tr>
<tr>
<td>Sow vaccination against atrophic rhinitis$^b$</td>
<td>No</td>
<td></td>
<td>1</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>0.91</td>
<td>2.48 (0.86-5.45)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Generalized $\chi^2$; Degrees of freedom 0.95.

$^b$ Variable was included in the final model, since the $P$ value was borderline not significant.
4. Discussion

The present study assessed the detection rate of *M. hyopneumoniae* in piglets of 3 weeks of age in different types of pig herds located in different European countries. The results showed that there was evidence of *M. hyopneumoniae* infection in 3-week old piglets in two thirds of the study herds. In most of the positive herds, the detection rate was between 1 and 10%. In some herds, more than 30% of the piglets were positive. This suggests that, in the selected herds, early infections with *M. hyopneumoniae* are important (Sibila et al., 2004, 2007; Fano et al., 2005). As specific criteria were used to select the herds (clinical problems in grower-finishing pigs attributed to this pathogen), the results may not be generalised to all pig herds within the EU, but only to herds meeting the selection criteria. However, in terms of management and housing conditions, the selected herds can be considered to be representative for many other pig herds in the EU (Sibila et al., 2004; Beilage et al., 2009).

Nested PCR on nasal swabs was used to analyse the samples. This diagnostic tool is suitable for detection of *M. hyopneumoniae* under field conditions in suckling pigs and nursery pigs (Otagiri et al., 2005; Sibila et al., 2007), detecting $5.1 \times 10^6$ ng *M. hyopneumoniae* DNA or as few as 4 organisms/μL reaction mixture (Gebruers et al., 2008). To avoid false positive results due to contamination in the nPCR, appropriate positive and negative controls were used during DNA extraction and amplification. The results should be interpreted at the herd level, not at the individual level (Otagiri et al., 2005).
M. hyopneumoniae attaches to and multiplies on the ciliated epithelium of the trachea, bronchi and bronchioles (Zielinski et al., 1993). Consequently, the nose is not the primary site of multiplication of M. hyopneumoniae organisms. It is therefore likely that the present results underestimate the number of infected animals in the selected herds and that a higher detection in recently weaned piglets would have been obtained from bronchoalveolar lavage fluid (BALF) (Moorkamp et al., 2009). Taking BAL fluid is possible under field conditions, but it is less practical and more time consuming than taking nasal swabs.

Serology would not work in the present study as it is not possible to discriminate between serum antibodies following infection and maternally derived antibodies. In addition, it takes at least 4-8 weeks before contact infected pigs may show detectable serum antibodies (Feld et al., 1992). Isolation of the bacterium is the gold standard for diagnosis of M. hyopneumoniae, but for the present study this method is inappropriate at herd level. Lung tissue or BAL fluid should be used instead of nasal swabs, nonetheless, these are very expensive, time consuming and special medium is required.

Although sow vaccination against M. hyopneumoniae is not commonly practiced, twenty eight percent of the sows in our study had been vaccinated before their first gestation. The detection rate of M. hyopneumoniae in the piglets was not significantly different between herds practicing sow vaccination and those not practicing vaccination. One could expect that vaccination in sows decreases the number of M. hyopneumoniae organisms shed by the sow and increases the level of maternal antibodies provided to the offspring. These factors could lead to a slight decrease in the
number of nPCR positive pigs at weaning (Sibila et al., 2007). The absence of a difference in the present study can be due to differences in vaccination strategies employed by the herds (e.g. timing of vaccination, age of the sows at vaccination, type of vaccine) and the large variability in serological response (30-100%) that may occur following sow vaccination (Thacker et al., 1998).

Herds vaccinating sows against SIV had a three times higher risk for having test-positive pigs compared to herds not vaccinating against SIV. From a biological point of view, it is not only unlikely, but also hard to explain that SIV vaccination would predispose to early *M. hyopneumoniae* infections. The significant and positive association may be due to the fact that herds with respiratory problems are more likely to vaccinate their sows against SIV. As most of the respiratory problems are due to multiple infections and also to non-infectious factors (Thacker et al., 2002), it is possible that in these herds, *M. hyopneumoniae* and/or other pathogens are involved in respiratory problems in addition to SIV.

These results indicate that, before a vaccination program is implemented, it is imperative to have a full picture of the problem in a herd and not to rely solely on the evaluation of the clinical symptoms. Performance of additional laboratory examinations (e.g. postmortem examination of dead animals, serology of different age groups) is required to investigate the different pathogens involved in the problem before establishing an aetiological diagnosis. Otherwise, vaccination programs may not be sufficient to control the main pathogen(s) in the herd.
Finally, some comments should be made with regard to the use of a cross-sectional study design and the causality of the risk factor. Only one group of piglets from each herd was sampled. Cross-sectional studies measure events at a particular time and therefore cause and effect are sometimes difficult to separate. The risk factor found in this study did not fulfil the criteria used to transpose observed associations into a causal relation (Susser, 1986). As already stated and explained, it was likely due to confounding (Thrusfield, 1997). Given that only one group of piglets was investigated within each herd, a sufficient number of piglets was tested and also a large number of herds from different European countries were included. This increases the validity of the results to other pig herds complying with the selection criteria.

This study showed that, using nested PCR on nasal swabs, *M. hyopneumoniae* infections in 3-weeks old pigs commonly occur in European pig herds suffering from respiratory disease in the grow-finishing pigs. No significant risk factors directly related to *M. hyopneumoniae* infections were found, but the significant variation in within-herd prevalence between herds indicates the need for further studies aiming to identify risk factors involved in the transmission of *M. hyopneumoniae* in suckling pigs. The results also reinforce the need to establish a precise and detailed diagnosis before implementing a vaccination scheme, as different pathogens may be involved in respiratory disease.
Acknowledgements

The farmers and herd veterinarians are acknowledged for collaboration with this study. Hanne Vereecke is acknowledged for excellent help in performing the laboratory work.
5. References


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3.2 Effect of vaccination against *Mycoplasma hyopneumoniae* on the transmission of *M. hyopneumoniae* under field conditions

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Abstract

This study investigated the effect of vaccination against *Mycoplasma hyopneumoniae* on its transmission in nursery pigs under field conditions. Seventy two pigs were randomly allocated at weaning into vaccinated (V) and non-vaccinated (NV) groups. Animals in the V group were vaccinated at 3 weeks-of-age with a commercial *M. hyopneumoniae* bacterin vaccine. Bronchoalveolar lavage fluid taken at weaning and at the end of the nursery period was assessed for the presence of *M. hyopneumoniae* by nested PCR, and the reproduction ratio of infection (*R*_n*) was calculated. The percentage of positive pigs in the V and NV groups was 14% and 36% at weaning, and 31% and 64% at the end of the nursery period, respectively. The *R*_n*-values for the V and NV groups were 0.71 and 0.56, respectively (*P* > 0.05). The study indicates that vaccination does not significantly reduce the transmission of this respiratory pathogen.
1. Introduction

*Mycoplasma hyopneumoniae* is the primary pathogen associated with enzootic pneumonia (EP) in pigs, a disease that causes significant economic loss to pig production worldwide (Thacker et al., 1999; Maes et al., 2003). *M. hyopneumoniae* is mainly transmitted through nose-to-nose contact, and indirectly via aerosol between susceptible pigs or through airborne transmission between farms (Goodwin, 1984, 1985; Thomson et al., 1992).

A sound understanding of the epidemiology and especially the transmission of the infection is a pre-requisite to optimal disease control. The transmission of this pathogen under experimental conditions has been quantified using a transmission ratio ($R_n$) (Meyns et al., 2006), and the values obtained indicated that vaccination did not significantly reduce pathogen transmission (Meyns et al., 2006). Similar studies quantifying transmission under ‘field’ conditions have not been carried out to the authors’ knowledge but are essential as transmission of infection under natural conditions may differ significantly from experimental settings. The nursery period in pig production is central to the transmission of *M. hyopneumoniae* (Clark et al., 1991; Sibila et al., 2009). At weaning, pigs infected during the suckling period are transferred to nursery units and mixed with susceptible non-infected animals, a process that facilitates the spread of pathogens such as *M. hyopneumoniae*.

Control of *M. hyopneumoniae* infection is largely achieved by improving management and production practices, by the use of antimicrobials and by vaccination (Maes et al., 2008). Vaccination with the commercially available bacterin vaccines is widely practiced and these vaccines are efficacious in reducing clinical signs, secondary bacterial respiratory infections, the
extent of lung lesions, and in improving production criteria (Thacker et al., 2004). However, experimental studies have shown that neither medication nor vaccination prevent colonisation of the respiratory tract by *M. hyopneumoniae* (Le Grand and Kobisch, 1996). As infection with this pathogen under natural conditions commonly occurs in weaned piglets (Sibila et al., 2009), it is important to assess if vaccination of suckling animals influences the extent of transmission of *M. hyopneumoniae* during the nursery period. The aim of this study was to quantify the transmission of *M. hyopneumoniae* in nursery pigs under field conditions, and to assess the effect of vaccination on this parameter. In addition, animal production criteria and the extent of lung lesions at slaughter were assessed.
2. Materials and methods

Details of herd under study

The study was conducted in 2008 in a Belgian pig herd comprising 1200 commercial hybrid sows (PIC) that operated a two-week batch production system. The sows were inseminated Piétrain semen. The reproductive performance of the sows (average number of live-born piglets, 11), and piglet health were good, although there had been some disease due to *Streptococcus suis* infection. The pre-weaning and nursery period mortality rates were approximately 9% and 2%, respectively. Piglets were cross-fostered during the first 3 days after birth and were weaned at 21 days-of-age. The piglets were given an iron injection at 2 days of age and were medicated with ceftiofur (Naxcel, Pfizer Animal Health) at 5 days-of-age in response to the *S. suis* infection.

Limited sampling using nasal swabs prior to the study indicated that 8/20 3 week-old piglets and 20/20 7 week-old piglets were positive for *M. hyopneumoniae* by nested (nPCR). No clinical signs of respiratory disease were evident in nursery piglets. The sows were vaccinated against *M. hyopneumoniae* (Porcilis Mhyo, Intervet Schering-Plough Animal Health) at 6 and 4 weeks prior to farrowing.

Experimental design

In total, 72 piglets were selected at weaning for study. Following standard management practice in the herd, all piglets were mixed after weaning and grouped into three weight categories (low, moderate and high weaning weight), based on visual assessment. The pigs used in the study were then randomly selected from the moderate weaning weight group. These animals were individually identified by ear-tag number, and randomly assigned to a vaccinated (V) \((n = 36)\) or
non-vaccinated (NV) \((n = 36)\) group. Pigs of the V group were vaccinated IM at 3 weeks-of-age with an inactivated \textit{M. hyopneumoniae} vaccine (Suvaxyn MH-One, Fort Dodge Animal Health). Pigs in the NV group were left untreated. Both groups were housed in separate nursery accommodation for 6 weeks after weaning. In each group all 36 animals were housed in one pen with solid partitions to eliminate nose-to-nose contact with other pigs in the same nursery room. The stocking density was 0.26 m\(^2\)/pig, the flooring was fully slatted, and the piglets were fed ad libitum with a commercial feed.

Each nursery room contained 16 pens (8 on each side of a central corridor) with approximately 36 pigs/pen. In consequence, the capacity of each nursery room was approximately 576 animals. In the nursery room containing the NV group, the pigs in the adjacent pens not involved in the trial were left unvaccinated, whereas those in the nursery room with the V group were vaccinated at 3 weeks-of-age. The rooms were mechanically ventilated via ceiling air inlets. At the end of the nursery period, the piglets were moved to a fattening unit located 1 km away. Here, the pigs under study were housed in four different pens in the same room with a capacity for approximately 144 pigs until they reached slaughter age (27 weeks). The pigs from the V group in the adjacent pens, were vaccinated at the start of the fattening period (Porcilis Mhyo, Intervet Schering-Plough Animal Health). The stocking density in the fattening unit was 0.77 m\(^2\)/pig.

\textit{Broncho-alveolar lavage sampling, nasal swabbing, nPCR analysis and bacteriological examination}

All 72 piglets were anaesthetised with 0.22 mL/kg of a mixture of xylazin (Xyl-M 2%, VMD) and zolazepam and tiletamin (Zoletil 100, Virbac) at 3 and 9 weeks-of-age to collect broncho-
alveolar lavage (BAL) fluid. Catheters with a diameter of 3.3 mm were used and 10 mL of phosphate buffered saline (PBS) was infused into the lungs and the fluid then gently aspirated. At 27 weeks-of-age, the lungs were removed at slaughter and immediately transported at 4 °C to the Faculty of Veterinary Medicine at Gent University. BAL was carried out on each right lung as described above except using 20 mL of PBS. The aspirated fluid was centrifuged at 4000 g for 30 min and the resulting pellet re-suspended in 1 mL of PBS and stored at –80 °C until DNA extraction was performed. DNA was extracted using a DNeasy blood and tissue kit (Qiagen), and a nPCR test was performed on the extract (Stärk et al., 1998). An animal was considered infected if the BAL fluid was found positive by nPCR.

Nasal swabs were collected at 3, 9 and 27 weeks-of-age from all animals. DNA was extracted with the DNeasy kit and nPCR was performed on the extract. A sample of each BAL fluid was inoculated onto Columbia and Columbia CNA agar each supplemented with 5% sheep blood (Oxoid). The Columbia agar also contained a *Staphylococcus intermedius* streak to support growth of *Actinobacillus and Haemophilus spp.*. Plates were incubated overnight in a 5% CO₂-enriched environment at 37 °C and isolated bacteria were identified (Quinn et al., 1994).

*Serological examination*

Blood samples were taken at 3 and 9 weeks-of-age, and at slaughter. Serological examination to detect antibodies against *M. hyopneumoniae*, was carried using an ELISA (DAKO Mh) (Feld et al., 1992). Sera with optical density (OD) values < 50% of the OD<sub>buffer-control</sub> were considered positive. OD values ≥ 50% of the OD<sub>buffer-control</sub> were classified as negative. Blood samples were also tested for antibodies against: porcine reproductive and respiratory disease virus (PRRSV)
(HerdChek PRRS, IDEXX); porcine circovirus type-2 (PCV2) (PCV2 ELISA, Shenzhen Lvshiyuan Biotechnology Co.); and porcine influenza sub-type H1N1 (HerdChek Swine Influenza virus, IDEXX).

Assessment of weight gain and of lung lesions

Pigs in the V and NV groups were individually weighed at 3, 9 and 26 weeks-of-age to determine average daily weight gain (ADG). ADG for these periods was estimated from the difference between the starting and final weight, divided by the number of days during that period. At slaughter, macroscopically visible pneumonia was quantified in a blinded fashion (Hannan et al., 1982). The scoring ranged from ‘0’ (no lesions) to 35 (entire lung affected). A Slaughterhouse Pleurisy Evaluation System (SPES) was used to grade any attendant pleurisy (Ostanello et al., 2007) and scores ranged from ‘0’ to ‘4’.

Statistical analysis

Estimation of the degree of inter-animal transmission of *M. hyopneumoniae* during the nursery period (when pigs were between 3 and 9 weeks-of-age) in each group was carried out using a stochastic infection model and by means of an adjusted reproduction ratio ($R_n$) (Meyns et al., 2004). In brief, this model assumes that the process of transmission between piglets is in accordance with the susceptible–infectious (S–I) model. This model was selected since it assumes that once an animal is infected, it does not recover before the end of the trial. At the start of the experiment, the number of *M. hyopneumoniae*-positive BAL fluid samples was determined by means of nPCR. The number of new contact infections was then determined by
the number of positive BAL fluid samples at the end of the trial, which in turn determines the scale of the outbreak. Based on this final number of infected animals, the number of infectious pigs at weaning (the start of the study), the transmission ratio can be estimated using the algorithm described by De Jong and Kimman (1994). This ratio is defined as the mean number of secondary cases caused by one infectious pig during the nursery period. The calculation of transmission ratio takes into account the different numbers of infected pigs in the V and NV groups at the start of the study (Kluivers et al., 2006). In this context, the different number of nPCR-positive animals at 3 weeks-of-age did not bias the $R_a$ values.

The prevalence of pigs seropositive for *M. hyopneumoniae* in the V and NV groups was analysed using logistic regression. Pig weights were compared between groups using a Student’s $t$ test for independent samples. Lung lesion scores were compared using a non-parametric Wilcoxon test. Result were considered statistically significant when $P < 0.05$ and data were analysed using SPSS 16.0 (SPSS Inc.).
3. Results

Detection of M. hyopneumoniae by nPCR in broncho-alveolar lavage fluid

The number of nPCR-positive BAL fluid samples at 3, 9 and 27 weeks-of-age are presented in Table 1. The percentage of positive samples at 3 weeks was 14% (5/36) and 36% (13/36) in the V and NV groups, respectively ($P > 0.05$). At 9 weeks of age, the percentage of positive samples in the V and NV groups was 31% (11/36) and 64% (23/36), respectively ($P < 0.05$). All pigs that tested positive at weaning remained positive at 9 weeks-of-age.

Of the nPCR-negative BAL fluid samples at 3 weeks-of-age ($n = 31$, V group; $n = 23$, NV group), 19% (6/31) were in the V group and 43% (10/23) of the NV group became positive at 9 weeks-of-age ($P = 0.05$). It follows that six and ten animals developed infection during the nursery period in the V and NV groups, respectively. The $R_n$ values (95% confidence interval) for the V and NV groups during the nursery period were estimated as $R_{nV} = 0.71$ (0.32, 2.06) and $R_{nN} = 0.56$ (0.29, 1.05), respectively ($P > 0.05$). For practical reasons, it was not possible to sample all the study pigs at slaughter so that 32 and 26 animals were sampled from the V and NV groups, respectively. The percentage of nPCR-positive pigs at slaughter was 59% and 7% in these groups, respectively ($P < 0.05$).

Detection of M. hyopneumoniae by nPCR in nasal swabs
All swabs from pigs at 3 weeks-of-age were negative on nPCR (Table 1). At 9 weeks-of-age, the percentage of nPCR-positive animals was 31% and 58% in the V and NV groups, respectively ($P < 0.05$). Nasal swabbing at slaughter was not carried out as such a procedure was likely to be adversely affected by carcase treatment procedures (Marois et al., 2008).

**Detection of M. hyopneumoniae by bacteriological culture of broncho-alveolar lavage fluid**

*S. suis* was the bacterium most frequently isolated from BAL fluid samples from both groups (Table 1). At 9 weeks-of-age, the prevalence of this pathogen was significantly lower in the V than in the NV group ($P < 0.05$). *Staphylococcus aureus* was isolated from BAL fluid samples in only one pig in the NV group at 3 weeks-of-age and at slaughter. *Actinobacillus pleuropneumoniae* was isolated from only one piglet in the V group at 3 weeks-of-age.

Table 1: Results of the nested PCR (nPCR) assessment of broncho-alveolar lavage (BAL) fluid and nasal swab samples, and of bacteriological examination of BAL fluid in pigs vaccinated (V) and not vaccinated (NV) against *Mycoplasma hyopneumoniae* at 3 weeks-of-age (weaning), 9 weeks-of-age (end of nursery phase of production) and at 27 weeks-of-age (slaughter). In total, 72 pigs from one herd were studied.
<table>
<thead>
<tr>
<th>Age of pigs at sampling</th>
<th>3 weeks (weaning)</th>
<th>9 weeks (end of nursery period)</th>
<th>27 weeks (slaughter) a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number (%) of nPCR-positive pigs on BAL fluid</strong></td>
<td>V</td>
<td>NV</td>
<td>P value</td>
</tr>
<tr>
<td>5/36 (14)</td>
<td>13/36 (36)</td>
<td>0.05</td>
<td>11/36 (31)</td>
</tr>
<tr>
<td><strong>Number (%) of nPCR-positive pigs on nasal swabs</strong></td>
<td>0/36 (0)</td>
<td>0/36 (0)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Number (%) of pigs positive for the following pathogens on bacteriological culture of BAL fluid:</strong></td>
<td>6/36 (17)</td>
<td>8/36 (22)</td>
<td>0.38</td>
</tr>
<tr>
<td><em>Streptococcus suis</em></td>
<td>0/36 (0)</td>
<td>1/36 (3)</td>
<td>0.50</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>0/36 (0)</td>
<td>1/36 (3)</td>
<td>0.50</td>
</tr>
<tr>
<td>b<em>A. pleuropneumoniae</em></td>
<td>0/36 (0)</td>
<td>1/36 (3)</td>
<td>0.50</td>
</tr>
</tbody>
</table>

a Not all pigs could be sampled at slaughter for practical reasons and nasal swabs were not taken at slaughter. b *A. pleuropneumoniae, Actinobacillus pleuropneumoniae.*
Serological examinations

The percentage of pigs seropositive for *M. hyopneumoniae* at 3 weeks-of-age was 33% and 14% in the V and NV groups, respectively (*P* > 0.05) (Table 2). At 9 weeks-of-age, the percentage of seropositive pigs in the V and NV groups was 42% and 3%, respectively (*P* < 0.05). At slaughter, the percentage of seropositive pigs in the V and NV groups was 56% and 35% (*P* > 0.05), respectively. The average serum OD values (±SD) for *M. hyopneumoniae* in the V and NV groups are detailed in Table 2. The percentage of pigs seropositive for PRRSV, PCV2 and H1N1 at 3, 9 and 27 weeks-of-age were not significantly different between the two groups (*P* > 0.05) (Table 2).

Table 2: Serological assessment and weights of pigs vaccinated (V) and not vaccinated (NV) against *Mycoplasma hyopneumoniae* at 3 weeks-of-age (weaning), 9 weeks-of-age (end of nursery phase of production) and at 26 weeks-of-age (one week prior to slaughter). In total, 72 pigs from one herd were studied. Animals were assessed for antibodies to *M. hyopneumoniae*, porcine reproductive and respiratory disease virus (PRRSV), porcine circovirus type-2 (PCV2), and porcine influenza sub-type H1N1.
### Age of pigs at sampling

<table>
<thead>
<tr>
<th>Age of pigs at sampling</th>
<th>3 weeks (weaning)</th>
<th>9 weeks (end of nursery period)</th>
<th>27 (slaughter) (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>NV</td>
<td><em>P</em> value</td>
<td>V</td>
</tr>
<tr>
<td>Number (% of pigs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>seropositive for *M.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>hyopneumoniae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12/36 (33)</td>
<td>10/36 (14)</td>
<td>0.40</td>
<td>15/36 (42)</td>
</tr>
<tr>
<td>Average OD (^b) value of sera for *M. <em>hyopneumoniae</em> (± SD)</td>
<td>69.4 (47.3)</td>
<td>74.6 (32.9)</td>
<td>0.67</td>
</tr>
<tr>
<td>Number (% of pigs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>seropositive for PRRSV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15/36 (41)</td>
<td>18/36 (50)</td>
<td>0.32</td>
<td>28/36 (78)</td>
</tr>
<tr>
<td>Number (% of pigs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>seropositive for PCV2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25/36 (69)</td>
<td>23/36 (64)</td>
<td>0.40</td>
<td>22/36 (61)</td>
</tr>
<tr>
<td>Number (% of pigs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>seropositive for porcine influenza (H1N1)</td>
<td>34/36 (94)</td>
<td>31/36 (86)</td>
<td>0.21</td>
</tr>
<tr>
<td>Average weight (± SD) (kg)</td>
<td>7.4 (1.0)</td>
<td>7.4 (0.9)</td>
<td>0.87</td>
</tr>
</tbody>
</table>

\(^a\) Not all pigs could be sampled at slaughter for practical reasons. \(^b\) OD, optical density.\(^c\) Fattening pigs were weighted at 26 weeks-of-age, one week prior to slaughter.
Average daily weight gain and extent of lung lesions

The weight of the pigs at 3, 9 and 27 weeks-of-age in the V and NV groups are detailed in Table 2. On average, pigs of the V group gained 5.1 kg more than pigs in the NV group between 3 and 26 weeks-of-age ($P > 0.05$). The ADG was significantly lower during the nursery period in the V group than in the NV group, whereas the opposite pertained during the fattening period (week 9 to 26) (Table 3). Over the entire period of the study, ADG was higher in the V than in the NV group ($581.9 \pm 55.7$ g/day versus $555.0 \pm 102.6$ g/day), although not significantly so ($P > 0.05$) (Table 3).

Table 3: Differences in average daily weight gain (ADG) (mean ± SD) between pigs vaccinated (V) and not vaccinated (NV) against *Mycoplasma hyopneumoniae* during the nursery period (3 to 9 weeks-of-age), the fattening period (9 to 26 weeks-of-age), and over the entire study (3 to 26 weeks-of-age). In total, 72 pigs from one herd were studied.

<table>
<thead>
<tr>
<th>Age range of (weeks)</th>
<th>ADG (g)</th>
<th>Difference in ADG (V-NV animals) (g)</th>
<th>Difference in ADG (%)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>NV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 to 9</td>
<td>217.3 ± 54.9</td>
<td>285.9 ± 57.9</td>
<td>-70.7</td>
<td>-24.0</td>
</tr>
<tr>
<td>9 to 26</td>
<td>667.1 ± 6.1</td>
<td>617.9 ± 2.7</td>
<td>+49.2</td>
<td>+7.9</td>
</tr>
<tr>
<td>3 to 26</td>
<td>581.9 ± 55.7</td>
<td>555.0 ± 102.6</td>
<td>+29.9</td>
<td>+4.8</td>
</tr>
</tbody>
</table>
The percentage of pigs with pneumonic lesions in the V and NV groups was 81 and 88%, respectively ($P > 0.05$) (Table 4). The average lesion scores in the V and NV groups was 0.97 and 0.71, respectively ($P > 0.05$). The average SPES score was $1.37 \pm 1.7$ for the V group and $0.67 \pm 0.9$ for the NV group, respectively ($P < 0.05$) (Table 4).

Table 4: Details of the extent of pneumonic lesions and pleuritis in pigs vaccinated (V) and not vaccinated (NV) against *Mycoplasma hyopneumoniae* at slaughter at 27 weeks-of-age. In total, 72 pigs from one herd were studied.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>NV</td>
</tr>
<tr>
<td>Number (%) of pigs with pneumonia</td>
<td>26/32 (81)</td>
<td>23/26 (88)</td>
</tr>
<tr>
<td>Average (± SD) pneumonia score</td>
<td>0.97 (1.5)</td>
<td>0.71 (0.9)</td>
</tr>
<tr>
<td>Number (%) of pigs with pleuritis</td>
<td>17/32 (53)</td>
<td>16/26 (62)</td>
</tr>
<tr>
<td>Average (± SD) pleuritis score</td>
<td>1.37 (1.7)</td>
<td>0.67 (0.9)</td>
</tr>
</tbody>
</table>
4. Discussion

This study has quantified the transmission of *M. hyopneumoniae* in nursery-phase pigs under field conditions, and has assessed the effect of single-dose vaccination on pathogen transmission. The work additionally investigated the prevalence of *M. hyopneumoniae* infection, animal performance during fattening and the severity of lung lesions at slaughter. The calculated $R_n$-values indicate that, during the six week nursery period, infected pigs infect on average 0.56 susceptible pen-mates. This value is lower than that previously found in transmission studies under experimental conditions (Meyns et al., 2004, 2006), and may be explained by factors such as stocking density, animal immune status and infection dose. The dose used to inoculate pigs in previous experimental studies (Meyns et al., 2004, 2006) was relatively high. Although the infecting dose under field conditions is not know, it would be expected to be lower, especially where good herd management and housing prevail, as in the present study. A lower infection dose is likely to result in lower numbers of organisms in the respiratory tract and thus a lower transmission rate and $R_n$ value.

Similar to previous experimental transmission studies (Meyns et al., 2004, 2006), the current work indicates that vaccination does not significantly reduce *M. hyopneumoniae* transmission under field conditions and suggests that vaccination alone will not eliminate infection with this pathogen from herds. The lack of any vaccination effect on transmission may have been due to the considerable numbers of pigs already infected at the time of vaccination at 3 weeks-of-age (14% in V and 36% in NV groups, respectively). The finding that pigs positive at vaccination remained positive at 9 weeks-of-age was not unexpected as vaccination of infected animals was not likely to result in the rapid clearance of *M. hyopneumoniae* from the lungs (Maes et al., 2008).
The 25% overall prevalence of infection at weaning in BAL fluid was higher than previously reported (Vicca et al., 2002; Sibila et al., 2007), although previous studies only carried out nPCR-detection on nasal swabs. Our results support those of a previous study that found more infected pigs on analysis of BAL fluid samples than of nasal swabs (Kurth et al., 2002), a finding particularly true of the 3 week-old piglets in our study. We chose to calculate the transmission ratio from the nPCR results from the BAL fluid and not from the nasal swabs, as *M. hyopneumoniae* is primarily found adherent to the ciliated epithelium of the trachea, bronchi and bronchioles rather than in the nasal chambers (Blanchard et al., 1992). Furthermore, although the assessment of nasal swabs may be suitable for investigating infection prevalence at a herd level, it is not appropriate in assessing the infection status of individual animals (Sibila et al., 2009). Nasal swabs were not taken at slaughter as contaminated water used in carcase treatment can reach the lungs, leading to the possibility of false positive results (Marois et al., 2008).

Given the high prevalence (88%) of lung lesions in the NV group at slaughter, it was surprising to find a low percentage of nPCR-positive results (7%) on analysis of BAL fluid from this group. Although the explanation for this remains unclear it is possible that the lungs of the NV animals contained greater amounts of DNA from other bacteria or from the blood within the lungs at slaughter. This DNA may not have been degraded fully during the extraction procedure and could have acted as an amplification inhibitor during nPCR (Baumeister et al., 1998). nPCR on BAL fluid from which the DNA is extracted using the QIAamp blood kit can be less sensitive in detecting *M. hyopneumoniae* when amplification inhibitors are not fully removed (I. Villarreal, personal communication).
The finding of a high prevalence of infected pigs at 3 weeks-of-age was somewhat surprising as the sows were vaccinated twice before farrowing. Vaccinating sows 5 and 3 weeks prior to farrowing induces high maternal antibody titres in suckling piglets and it is associated with lower numbers of infected animals at weaning as detected by nPCR on nasal swabs (Ruiz et al., 2003; Sibila et al., 2007, 2008a). The higher number of seropositive piglets at 3 weeks-of-age, compared to 9 weeks-of-age, was due to the effect of maternal antibody. At 3 weeks-of-age, the percentage of seropositive pigs was higher in the V (33%) than in the NV (14%) group and the average OD values were slightly lower (69.4 vs. 74.6). It remains unknown if these differences could have accounted for the lower infection prevalence found by nPCR on BAL fluid samples from the V group at this age. Maternally-derived antibodies, under experimental conditions, provide limited to no effect on respiratory tract colonisation by *M. hyopneumoniae* (Thacker et al., 2000). Since our transmission ratios take into account the numbers of infected pigs in both groups at the start of the study, the different numbers of nPCR-positive animals at 3 weeks-of-age have not biased these calculations.

The influence of maternal antibody on the vaccine response in the present study is not clear. As the efficacy of vaccination of suckling pigs has been demonstrated under both experimental and field conditions (Jensen et al., 2002), it is unlikely that such antibodies significantly influenced the host response to vaccination. Antibody levels can remain the same (Martelli et al., 2006), or be reduced (Maes et al., 1999; Hodgins et al., 2004) following the vaccination of pigs with high levels of maternally-derived antibody. Thus as the optimal timing of vaccination must balance the advantages of delayed vaccination with the need to induce adequate early immunity, and given the considerable number of infected pigs we found at weaning, the early vaccination strategy we adopted in this study appears justified.
The reason for the significantly lower ADG during the nursery phase of production in the V than in the NV group is not clear. However, during the fattening phase and by 26 weeks-of-age, the ADG was significantly higher in the V than in the NV pigs. This finding is in agreement with previous studies (Jensen et al., 2002). Although studies show that a single-dose vaccination protocol can decrease the incidence of clinical disease and of lung lesions at slaughter (Dawson et al., 2002; Moreau et al., 2004), in the present study no significant difference in the percentage of pigs with pneumonic lesions or in the severity of these lesions was found between the groups. The reason for this finding is not known, but it may be due to: the small study population; the effects of re-infection; the fact that vaccinated and non-vaccinated animals were housed in the same accommodation during the fattening period; or the absence of significant clinical disease in this case. Our findings are consistent with those of a previous study (Vicca et al., 2002), where a high prevalence of infection, even at a young age, did not necessarily correlate with a higher incidence of clinical disease or with more substantial lung lesions at slaughter. The lack of clinical disease experienced in our study herd may be attributed to the good management and housing conditions pertaining, and also to the fact that the strain of *M. hyopneumoniae* circulating in this herd was of low virulence (Vicca et al., 2003).

This field study has found that, similar to previous transmission studies of *M. hyopneumoniae* under experimental conditions (Meyns et al., 2006), vaccination with a bacterin-based vaccine does not significantly reduce the transmission of this pathogen between pigs. This finding implies that vaccination alone will not be effective in eliminating *M. hyopneumoniae* infection from herds.
Acknowledgements

This research was financially supported by Fort Dodge Animal Health. The authors are grateful to the herd-owner for their collaboration as well as to Hanne Vereecke for laboratory support.
5. References


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3: EXPERIMENTAL STUDIES


Protection against different strains of *Mycoplasma hyopneumoniae*
3.3 Effect of vaccination of pigs against experimental infection with highly and low virulent *Mycoplasma hyopneumoniae* strains

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Vaccine, Submitted Oct, 2010
Abstract

This study investigated the infection pattern and lung lesion development in pigs caused by a low and highly virulent *M. hyopneumoniae* strain at 4 and 8 weeks (w) post infection (PI). It also determined the efficacy of a commercial inactivated whole-cell vaccine against infection with each one of these *M. hyopneumoniae* strains. Ninety piglets free of *M. hyopneumoniae* were selected, and 40 of them were randomly vaccinated during their first week of life. At weaning, all piglets were allocated to 10 different groups and housed in pens with absolute filters. At 4 weeks of age, pigs were inoculated intratracheally with either a highly virulent *M. hyopneumoniae* strain, a low virulent strain or with sterile culture medium. Half of all animals were euthanized at 4w PI, while the remaining half was euthanized at 8w PI. Coughing was assessed daily, and lung lesions, immunofluorescence (IF), bacteriological analysis and nested PCR were assessed after necropsy. It was demonstrated that contrary to the highly virulent strain, the low virulent strain required more than 4 weeks PI (commonly accepted as the standard infection model) to reach maximum clinical symptoms. Vaccination significantly reduced clinical symptoms, macroscopic and microscopic lung lesions in pigs infected with the highly virulent strain. This effect was more pronounced at 4 than at 8 weeks PI. Protective efficacy was also observed in pigs infected with the low virulent strain, but the effect was less pronounced than with the highly virulent strain.
1. Introduction

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the causative agent of enzootic pneumonia in swine, a disease with worldwide economic impact in intensive pig production (Thacker et al., 1999; Maes et al., 2008) and characterized by chronic non productive coughing, retarded growth rate and poor feed conversion ratio (Ross 1992).

It is well known that factors such as management practices and housing conditions influence the infection pattern and the severity of the disease in pig herds (Maes et al 1999). However, also the virulence of *M. hyopneumoniae* strains has been demonstrated to determine the clinical course of the infection (Vicca et al., 2003). Experimental infection with a highly virulent *M. hyopneumoniae* strain was associated with severe clinical signs and lung lesions 4 weeks post infection, whereas clinical symptoms and lung lesions were milder following infection with low virulent strains (Meyns et al., 2006; Vicca et al., 2003).

In most *M. hyopneumoniae* experimental infection studies, pigs are euthanized at 4 weeks post infection (PI), based on the fact that macroscopic lung lesions reach their maximum size 2-4 weeks after inoculation (Whittlestone et al., 1972; Kobisch et al., 1993). This model has been widely accepted as the standard infection model for this pathogen. However, recent experiments showed that, depending on the strain, the most severe clinical symptoms and lung lesions are sometimes seen later than 4 weeks after infection (Villarreal et al., 2009). As a result, the standard infection model might not be suitable for studying all types of *M. hyopneumoniae* strains, as some of them might require more than 4 weeks PI to fully develop symptoms and lesions in the infected pigs.

Vaccination is frequently practiced to control *M. hyopneumoniae* infections in pig herds worldwide. Although vaccination with the currently available bacterin vaccines is not able to prevent transmission nor establishment of the microorganism in the lungs (Meyns et al., 2006;
Thacker et al. 1998), numerous studies have shown that it reduces the clinical symptoms and lung lesions, improves performance, and leads to an economic benefit for the pig producers (Maes et al., 2003; Moreau at al., 2004; Maes et al., 2008; Sibila et al., 2007). *M. hyopneumoniae* is, however, a highly heterogenous species not only with regard to virulence (Meyns et al., 2006; Vicca et al., 2003), but also at genetic, antigenic and proteomic level (Calus et al., 2010; Pinto et al., 2009; Stakenborg et al., 2005). Most of the currently available bacterin vaccines are based on an adjuvanted suspension of inactivated *M. hyopneumoniae* organisms, and it is not clear whether the protective effect of vaccination may vary depending on the strain causing the infection.

Therefore, the aims of this study were twofold. First, in order to assess the suitability of the standard infection model, the present study investigated the infection pattern and lung lesion development in pigs infected by low and highly virulent *M. hyopneumoniae* strains during 8 weeks after infection. Secondly, the effect of vaccination with a commercial vaccine against infection with *M. hyopneumoniae* strains of low and high virulence was evaluated.
2. Materials and methods

M. hyopneumoniae strains and intratracheal inoculation procedure

For experimental infection of pigs, the low virulent strain (F13.7B) and the highly virulent strain (F7.2C) were used (Vicca et al., 2003; Meyns et al., 2007). Both strains have been differentiated at a genomic and proteomic level as described by Stakenborg et al., (2006) and Calus et al., (2007).

For experimental infection, pigs were anesthetized with 0.22 ml/kg of a mixture of Xyl-M® 2% (VMD, Arendonk, Belgium) and Zoletil 100® (Virbac, Louvain la Neuve, Belgium), and they were inoculated intratracheally with $7 \times 10^7$ CCU/ml of either the low or highly virulent M. hyopneumoniae strain in 7 ml inoculum, or with 7 ml of sterile culture medium (Friis, 1975).

Experimental design

The experimental design is summarized in Table 1.

Ninety cross-bred piglets free of M. hyopneumoniae and PRRSV were included in the study. All animals were obtained from a herd that has been free of M. hyopneumoniae and PRRSV for more than 15 years based on repeated serological testing, and absence of clinical symptoms and pneumonia lesions. All pigs were selected at one week of age, 40 of them were randomly vaccinated (v) on the farm with a one-shot commercial vaccine (Stellamune® One, Pfizer Animal Health). Stellamune is a whole-cell bacterin based on strain P-5722-3 (NL 1042), inactivated with binary ethylamine and oil adjuvanted. Animals were administered 2 ml of the commercial vaccine. The non vaccinated animals were injected with 2 ml of phosphate buffered saline (PBS), used as a placebo.
At 21 days of age, piglets were weaned and moved to the experimental facilities of the Faculty of Veterinary Medicine, Ghent University, Belgium. They were housed in pens with absolute filters (HEPA U15) and fed *ad libitum* a commercial feed without antimicrobials.

After one week of acclimatization, the vaccinated piglets were randomly allocated to four groups. Two groups were intratracheally challenged with the low virulent strain (vLV-4 and vLV-8) and two with the highly virulent strain (vHV-4 and vHV-8). Forty non-vaccinated piglets (nv) (n=40) were also assigned to four groups namely two intratracheally challenged with the low virulent strain (nvLV-4 and nvLV-8) and two with the highly virulent strain (nvHV-4 and nvHV-8). The remaining ten non-vaccinated piglets were sham-challenged with sterile culture medium and allocated to groups nvNC-4 and nvNC-8, used as negative controls.

At 4 weeks post intratracheal inoculation (PI), half of the total number of piglets was euthanized, whereas the remaining half was euthanized at 8 weeks PI. Deep anaesthesia was applied prior to euthanasia by administrating intramuscularly 0.3 ml/kg of a mixture of XylM® 2% and Zoletil 100®, followed by exsanguination. The numbers 4 and 8 in each group mentioned above designate the weeks (w) PI when piglets were euthanized. The study was performed after approval of the Ethical Committee for Animal Experiments of the Faculty of Veterinary Medicine, Ghent University.

*Clinical parameters*

After intratracheal inoculation, piglets were observed daily for a minimum of 15 minutes. The severity of coughing was assessed using a respiratory disease score (RDS) as described by Halbur et al. (1996). Body condition, appetite, presence of dyspnea and tachypnea were also recorded. The RDS could range from 0 to 6: 0 (no coughing), 1 (mild coughing after an
encouraged move), 2 (mild coughing in rest), 3 (moderate coughing after encouraged move),
4 (moderate coughing in rest), 5 (severe coughing after encouraged move), 6 (severe coughing
in rest). The daily RDS values were summed and averaged for the two periods namely D28-
D56 (the first 4 weeks PI) and D56-D84 (the last 4 weeks PI).

Serology
At the age of 7, 28, 42, 56, 70 and 84 days, blood samples were taken from all surviving pigs
and tested for the presence of antibodies against *M. hyopneumoniae* using the DAKO® Mh
ELISA (Dako Cytomation, Denmark) (Feld *et al.*, 1992). Sera with optical density (OD)-
values < 50% of the OD_{buffer-control} were considered positive, OD-values > 50% of the OD_{buffer-
control} were classified as negative. On day 28, blood samples were also checked for PRRSV
serum antibodies by means of the HerdChek® PRRS ELISA (Idexx Laboratories, Westbrook,
ME, USA).

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

After necropsy, the lungs were removed to score the macroscopic pneumonia lesions
according to Hannan *et al.* (1982), with values ranging from 0 (no lesions) to 35 (entire lung
affected).

Samples from the right apical, cardial and diaphragmatic lung lobe of each pig were collected
for histopathological examination and IF testing. If lung lesions were present, tissue samples
for IF testing were collected from the edge of a lesion.
For histopathological examination, the lung tissue samples were fixed in 10% neutral formalin, processed and embedded in paraffin. After hematoxylin and eosin staining, samples were scored using light microscopy. Scores could range from 0 to 5, according to the degree of peribronchiolar and perivascular lymphohistiocytic infiltration, as well as cuffing formation (Morris et al., 1995; Villarreal et al., 2009). Scores 1 (limited cellular infiltrates—macrophages and lymphocyte- around bronchioles; with airways and alveolar spaces free of cellular exudates) and 2 (moderate infiltrates with mild diffuse cellular exudates into airways) were classified as lesions not related to *Mycoplasma* infections. Scores 3, 4, 5 were considered to be associated with *Mycoplasma* infection (mild, moderate or severe- broncho-interstitial (cuffing) pneumonia, surrounding bronchioles but extending to the interstitium, with lymphofollicular infiltration and mixed inflammatory cell exudates). The average histopathological lung score per group was calculated.

A direct IF assay (Kobisch et al., 1978) was performed to assess the load of *M. hyopneumoniae* organisms in a semi-quantitative way. The scores could range from 0 to 3: 0 (no IF), 1 (limited IF), 2 (moderate IF), and 3 (intense IF) (Vicca et al., 2003).

**Nasal swabs, bronchoalveolar lavage fluid, and nPCR**

Nasal swabs were collected from all animals just before challenge on day 28 and at euthanasia (on day 56 and 84).

At necropsy (day 56 or 84), 50 ml of Friis medium was slowly infused in the left lung and aspirated back into the syringe. The recovered bronchoalveolar lavage (BAL) fluid was aliquoted, immediately cooled at 4°C, and subsequently stored at -70°C until analysis.

DNA was extracted from the nasal swabs and BAL fluid with the QIAGEN protocol for purification of total DNA from animal blood and tissues (QIAGEN, DNeasy Blood & Tissue
Kit, Belgium). For detection of *M. hyopneumoniae* DNA, a two steps nPCR-test based on a modified protocol described by Stärk et al. (1998) was performed (Villarreal et al., 2009). Briefly the nested PCR was performed using *Taq* polymerase (GIBCO Invitrogen, Belgium) in a final volume of 20 µl for the first step and 15 µl for the second step. Reaction mixtures contained PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 3 mM MgCl₂, 180 µM of each dNTP, 0.5 U *Taq* polymerase and 0.25 µM of each primer MHP950-1L (5’ AggAACACCATCgCgATTTTTA 3’) and MHP950-1R (5’ ATAAAAATggCATTCCTTTTCA 3’) (first step) or MHP950-2L (5’ CCCTTTgTCTTAATTTTTgCAA3’) and MHP950-2R (5’ gCCgATTCTAgTACCCTAATCC 3’) (second step). Two µl of template DNA was used in the first step, 1.5 µl of amplification product of the first step was used as template in the second step. For both steps, the following PCR program was used: initial denaturation at 94°C for 2 min; denaturation at 94°C for 30 s, annealing at 51°C for 30s, and extension at 72°C for 1 min; 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min; final extension at 72°C for 5 min followed by cooling to 10°C. Nested PCR products were analyzed by electrophoresis on 1.5 % agarose gels in TBE buffer and visualized under UV illumination after staining in GelRed™ (Biotium. Inc., CA. USA).

**Bacteriology**

A sample of BAL fluid per pig in each group was inoculated on Columbia agar supplemented with 5% sheep blood (Oxoid, Hampshire, UK) with a *Staphylococcus intermedius* streak for support of *Actinobacillus* and *Haemophilus* sp. growth, and on Columbia CNA agar with 5% sheep blood (Oxoid, Hampshire, UK). As described by Quinn et al. (1994), plates were incubated overnight in a 5% CO₂-enriched environment at 35°C for 48 hours for identification of respiratory bacteria in the lungs.
Statistical analysis

One-way analysis of variance (ANOVA) was used to analyse RDS scores, macroscopic and histopathological lung lesions and IF testing. Levene’s test was used to assess the homogeneity of the variances between the different groups. For each parameter, Bonferroni’s pair-wise comparisons test was used to evaluate possible differences between groups. This comparison was made for the two periods 28-56 days (4 weeks PI) and 56-84 days (8 weeks PI). Statistical results were considered significant when P-values were ≤0.05. The statistical package SPSS 17.0 for Windows (SPSS 15, SPSS Inc. Illinois, USA) was used to analyse the data.
3. Results

Clinical parameters

Three out of the 90 piglets died during the intratracheal inoculation: one from the control group nvNC-4, one from the nvHV-4 group and one from nvHV-8 group. Necropsies were performed, but no pathological abnormalities were diagnosed.

The average RDS for the different groups is presented in Table 1. Throughout the entire trial, coughing was absent in the nvNC-4 and nvNC-8 groups. The onset of coughing in groups nvLV-4 and nvLV-8 started later (25 DPI) and was milder than in the groups nvHV-4 and nvHV-8, for which coughing started at ±12 DPI. In the vaccinated groups, the onset of coughing was observed around the same period as in the corresponding non vaccinated groups. However, the overall severity of coughing was lower compared to the non vaccinated groups infected with the same strains.

During the first 4 weeks PI, the highest average RDS value was observed for the nvHV-4 group, which significantly differed from the other groups (P<0.05).

During the 8th week PI, the average RDS values in groups nvLV-8, nvHV-8, vLV-8 and nvHV-8, were 0.24, 0.44, 0.05 and 0.30, respectively. Significant difference (P<0.05) was found between the vaccinated groups (vLV-8 and vHV-8) in relation to the respective non vaccinated groups (nvLV-8 and nvHV-8).

Serology

None of the piglets was serologically positive for M. hyopneumoniae at 7 days of age. The piglets of the nvNC-4 and nvNC-8 remained negative throughout the study (Table 2).
In the non vaccinated groups infected with the highly virulent strain, nvHV-4 and nvHV-8, the first seroconverted pigs were detected on day 42. In the non vaccinated groups infected with the low virulent strain, nvLV-4 and nvLV-8, seroconverted pigs were detected later (day 56). By the end of the study on day 84, nearly 90% of the animals belonging to the non vaccinated groups were serologically positive.

In the vaccinated groups, vHV-4, vHV-8 and vLV-4, vLV-8, 100% of the piglets were serologically positive three weeks after vaccination and remained positive until the day of euthanasia.

Macroscopic lung lesions, histopathological findings and results of IF testing for M. hyopneumonia

Macroscopic lung lesions were not observed in the negative control groups, nvNC-4 and nvNC-8. At 4 weeks PI, significant differences (P<0.05) were found between the different groups (Table 1). The highest mean values for macroscopic lesions were observed in group nvHV-4 (6.69), followed by nvLV-4 (2.38); the lowest were in groups vHV-4 (0.11) and vLV-4 (0.93). At 8 weeks PI, the highest macroscopic lung lesion score was recorded for group nvHV-8 (5.60), which was significantly different (P<0.05) from the remaining groups: vHV-8 (1.99), vLV-8 (1.74) and nvLV-8 (1.20).

The mean scores of the histopathological lesions in the different groups at 4 weeks PI were, from highest to lowest, 4.04 (nvHV-4), 3.10 (vLV-4), 3.02 (vHV-4) and 2.90 (nvLV-4). The mean score of the nvNC-4 group (1.17) was significantly lower than the mean scores of all other groups (P<0.05), whereas the mean score of the nvHV-4 group was significantly higher (P<0.05) than the scores of the other groups (Table 1). At 8 weeks PI, the mean histopathological scores were 3.62 (nvHV-8), 3.41 (vLV-8), 3.35 (vHV-8) and 2.92 (nvLV-
There were no significant differences between groups, apart from the score in the nvNC-8 group, which was the lowest (1.17) (P<0.05).

Apart from the negative control groups, nvNC-4 and nvNC-8, all pigs were positive for IF staining (Table 2). At 4 weeks PI, there were no statistical differences between vaccinated and non-vaccinated groups. The highest mean IF score was present in the nvHV-4 (1.26). At 8 weeks PI, the highest IF mean score was recorded in group nvHV-8 (1.44). Group vHV-8 obtained a significantly lower IF score than group nvHV-8 (P< 0.05).

*nPCR testing for M. hyopneumoniae on BAL fluid and nasal swabs*

The results of the nPCR on BAL fluid and nasal swabs are presented in Table 1. Both non infected control groups, nvNC-4 and nvNC-8, were negative for nPCR testing on BAL fluid and nasal swabs. For the other groups, presence of *M. hyopneumoniae* was confirmed at both 4 and 8 weeks PI.

*Bacteriology*

At 4 weeks PI, *Bordetella bronchiseptica* was the only bacterium isolated from BAL fluid in animals from groups nvLV-4 (3/10), nvHV-4 (1/9), vLV-4 (2/10) and vHV-4 (3/10). At 8 weeks PI, *Bordetella bronchiseptica* was isolated only in groups nvHV-8 (1/9) and vHV-8 (3/10). *Haemophilus parasuis* was also isolated from one animal in group nvLV-8.
Table 1: Clinical, (histo)-pathological observations and presence of *M. hyopneumoniae* antigens or DNA in the respiratory tract of pigs intratracheally inoculated with sterile culture medium, a highly or low virulent *M. hyopneumoniae* strain.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Nb(^1) pigs</th>
<th>Vaccinated(^2)</th>
<th>Inoculated(^3) with</th>
<th>Necropsy at (weeks PI(^4))</th>
<th>Average RDS(^5)</th>
<th>Average lung lesion score</th>
<th>Average IF(^6) score</th>
<th>nPCR(^7)</th>
<th>Macrosc.</th>
<th>Microsc.</th>
<th>BALF(^8)</th>
<th>Nasal swabs</th>
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<td>nvLV-4</td>
<td>10</td>
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<td>LV</td>
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<td>0.07(^a)</td>
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<td>2.90(^b)</td>
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<tr>
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<td>No</td>
<td>LV</td>
<td>8</td>
<td>0.24(^{b,d})</td>
<td>1.20(^A)</td>
<td>2.92(^B)</td>
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<td>HV</td>
<td>4</td>
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<td>HV</td>
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<td>10</td>
<td>Yes</td>
<td>HV</td>
<td>8</td>
<td>0.30(^{C,D})</td>
<td>1.99(^A)</td>
<td>3.35(^B)</td>
<td>0.67(^{A,C})</td>
<td>10</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nvNC-4</td>
<td>5</td>
<td>-</td>
<td>medium</td>
<td>4</td>
<td>0.00(^a)</td>
<td>0.00(^a)</td>
<td>1.17(^a)</td>
<td>0.00(^a)</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nvNC-8</td>
<td>5</td>
<td>-</td>
<td>medium</td>
<td>8</td>
<td>0.00(^A)</td>
<td>0.00(^A)</td>
<td>1.17(^A)</td>
<td>0.00(^A)</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different lowercase letters within a column correspond to significantly different values between the groups at 4w PI; different capital letters within a column indicate significantly different values between the groups at 8w PI (P< 0.05).
Animals were vaccinated at 7 days of age with an inactivated M. hyopneumoniae whole-cell vaccine

Animals were intratracheally inoculated at 28 days of age with either sterile culture medium, a highly virulent (HV) or low virulent (LV) M. hyopneumoniae strain.

PI: post-infection

RDS: Respiratory disease score

IF: Immunofluorescence

Number of positive samples by nPCR

BALF: bronchoalveolar lavage fluid
Table 2: Percentage of seropositive pigs (average OD-values) in the different groups at different time-points during the experimental study. Sera with optical density (OD)-values < 50% of the OD$_{\text{buffer-control}}$ were considered positive, OD-values > 50% of the OD$_{\text{buffer-control}}$ were classified as negative.

<table>
<thead>
<tr>
<th>Age of piglets</th>
<th>Group</th>
<th>D7</th>
<th>D28</th>
<th>D42</th>
<th>D56</th>
<th>D70</th>
<th>D84</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nvLV-4</td>
<td>0 (93.4)</td>
<td>0 (80.9)</td>
<td>0 (52.0)</td>
<td>50 (43.4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>nvLV-8</td>
<td>0 (84.4)</td>
<td>0 (85.9)</td>
<td>0 (51.0)</td>
<td>50 (35.7)</td>
<td>80 (56.6)</td>
<td>90 (56.3)</td>
</tr>
<tr>
<td></td>
<td>nvHV-4</td>
<td>0 (86.5)</td>
<td>0 (85.9)</td>
<td>11 (21.5)</td>
<td>89 (67.8)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>nvHV-8</td>
<td>0 (84.2)</td>
<td>0 (78.7)</td>
<td>11 (16.1)</td>
<td>78 (47.9)</td>
<td>89 (63.4)</td>
<td>80 (62.4)</td>
</tr>
<tr>
<td></td>
<td>vLV-4</td>
<td>0 (82.2)</td>
<td>90 (43.6)</td>
<td>90 (75.1)</td>
<td>90 (93.9)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>vLV-8</td>
<td>0 (73.2)</td>
<td>100 (39.9)</td>
<td>100 (76.9)</td>
<td>100 (101)</td>
<td>100 (104)</td>
<td>100 (107)</td>
</tr>
<tr>
<td></td>
<td>vHV-4</td>
<td>0 (82.7)</td>
<td>100 (22.1)</td>
<td>100 (74.1)</td>
<td>100 (108)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>vHV-8</td>
<td>0 (82.7)</td>
<td>100 (20.2)</td>
<td>100 (80.8)</td>
<td>100 (108)</td>
<td>100 (108)</td>
<td>100 (109)</td>
</tr>
<tr>
<td></td>
<td>nvNC-4</td>
<td>0 (84.0)</td>
<td>0 (72.3)</td>
<td>0 (13.3)</td>
<td>0 (22.4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>nvNC-8</td>
<td>0 (88.4)</td>
<td>0 (81.3)</td>
<td>0 (6.9)</td>
<td>0 (16.6)</td>
<td>0 (19.7)</td>
<td>0 (20.1)</td>
</tr>
</tbody>
</table>
4. Discussion

In corroboration with past findings (Meyns et al., 2007; Vicca et al., 2003), the clinical symptoms and lung lesions induced by the low virulent *M. hyopneumoniae* strain were milder than those caused by the highly virulent strain. Most experimental trials monitor clinical symptoms and lung lesions only during 4 weeks after challenge. Our results suggest that although macroscopic lung lesions reached their maximal score within 4 weeks after challenge, the average RDS in pigs infected with the low virulent strain were higher at 8 weeks than at 4 weeks PI. Thus, a longer period should be considered when studying clinical symptoms caused by low virulent strains. The understanding of variations in infection patterns between individual *M. hyopneumoniae* strains is important for the implementation of better control measures offering protection against different strains.

The non vaccinated pigs infected with the low virulent strain (nvLV-4 and nvLV-8) had a slower and lower seroconversion rate than those infected with the highly virulent strain (nvHV-4 and nvHV-8), very similar to the results obtained by Meyns et al. (2004) in a transmission study with the same strains. In our trial, two weeks after challenge (42 days of age), 11% of the pigs belonging to the groups infected with the highly virulent strain had seroconverted, compared to 0% seroconversion in the groups infected with the low virulent strain (P< 0.05). The exact reasons for the faster seroconversion in pigs challenged with the highly virulent strain are not known. It could be due to faster multiplication and induction of a more severe inflammatory process in the lungs by this strain (Meyns et al., 2007).

All vaccinated pigs (vLV-4, vLV-8, vHV-4 and vHV-8) had seroconverted within three weeks after vaccination. These results are in agreement with other studies (Sibila et al., 2007), where a one-shot vaccine during the first week of life induced 100% seroconversion by the 25th day of age.
In the present study, more positive pigs were detected in BALF samples than in nasal swabs, confirming that nested PCR on BALF is more suitable as a tool to test infection in individual animals (Kurth et al., 2002; Sibila et al., 2009).

Vaccination delayed the clinical onset of disease (coughing) and overall significantly reduced its severity, both in pigs infected with the highly virulent strain and in those infected with the low virulent strain. The reduction in clinical disease and macroscopic lung lesions was, however, more prominent in pigs challenged with the highly virulent strain than in pigs challenged with the low virulent strain. This might be due to the fact that the low virulent strain only causes mild clinical symptoms and lung lesions are less severe. Hence, the threshold for improvement by vaccination is lower. At 8 weeks PI, however, significantly lower IF scores were only demonstrated in vaccinated pigs challenged with the highly virulent strain and not in the vaccinated animals challenged with the low virulent strain. This might indicate that the capacity of the vaccine used in the present study to suppress *M. hyopneumoniae* multiplication in the lungs is higher after infection with the highly virulent strain. In the field, variation in efficacy of vaccination strategies between different herds has also been reported (Maes et al., 1999) and it has been suggested that differences in virulence as well as divergent protein variability between circulating *M. hyopneumoniae* strains may be involved (Calus et al., 2007, Vicca et al., 2003).

In conclusion, the current study demonstrated a difference in infection pattern between the two *M. hyopneumoniae* strains both at 4 and 8 weeks PI. Contrary to the highly virulent strain, the low virulent strain required a period longer than 4 weeks post infection (commonly accepted as the standard infection model) to induce maximum clinical symptoms. In addition, vaccination significantly reduced clinical symptoms, macroscopic and microscopic lung lesions in pigs infected with the highly virulent strain. Protective efficacy was also observed in pigs infected with the low virulent strain, but the effect was less pronounced.
Acknowledgements

The study was financially supported by IWT project number 050642. The authors would like to thank Hanne Vereecke and Alfonso Lopez for all the help and effort dedicated to the project.
5. References


3.4 Infection with a low virulent *Mycoplasma hyopneumoniae* isolate does not protect piglets against subsequent infection with a highly virulent *M. hyopneumoniae* isolate

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Vaccine, 27, 2009 (12): 1875-1879
Abstract

The study aimed to evaluate the effect of an infection with low virulent isolates of *M. hyopneumoniae* (LV1 and LV2) on the subsequent infection with a highly virulent isolate (HV). Fifty-five, 3 week-old piglets free of *M. hyopneumoniae* were randomly allocated to 6 different groups. At 4 weeks of age (D0), groups LV1-HV and LV1 were intratracheally inoculated with LV1, groups LV2-HV and LV2 with LV2, and group HV with sterile culture medium. Four weeks later (D28), the pigs of these different groups were either intratracheally inoculated with the highly virulent isolate (groups LV1-HV, LV2-HV, HV) or with sterile culture medium (groups LV1 and LV2). A negative control group consisted of pigs inoculated with sterile culture medium on D0 and D28. All animals were necropsied at 28 days after the second inoculation (D56). Clinical symptoms were evaluated daily using a respiratory disease score (RDS). After necropsy, macroscopic and histopathological lung lesions were quantified and immunofluorescence (IF) testing on lung tissue and nested PCR on BAL fluid were performed for the detection of *M. hyopneumoniae*.

Disease signs and lung lesions were not observed in pigs of the negative control group. In the other groups, there were no or only very mild clinical symptoms from D0 until D28. A significant increase in the average RDS values was, however, observed during D28-D56, especially in groups LV1-HV (1.48) and LV2-HV (1.49), in group HV (0.79), and to a lesser extent in groups LV1 (0.50) and LV2 (0.65) (P<0.05). The clinical symptoms during D28-D56, the lung lesions and intensity of IF staining were more pronounced in groups LV1-HV, LV2-HV and HV compared to groups LV1 and LV2. All pigs, except those from the negative control group, were positive on IF testing and PCR at D56. The present study demonstrates that pigs inoculated with low virulent isolates of *M. hyopneumoniae* are not protected against a subsequent infection with a highly virulent isolate 4 weeks later and may even develop more
severe disease signs. This indicates that subsequent infections with different *M. hyopneumoniae* isolates may lead to more severe clinical disease in a pig herd.
1. Introduction

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the primary cause of enzootic pneumonia in pigs, a chronic respiratory disease that causes major losses to the pig industry worldwide (Thacker et al., 1999). The infection pattern and the severity of the disease are largely determined by management practices and housing conditions (Maes et al., 2008), but also the virulence of the *M. hyopneumoniae* isolate is important (Vicca et al., 2003; Meyns et al., 2004). Experimental infections with a highly virulent isolate consistently resulted in clinical disease and lung lesions, whereas infection with a low virulent isolate was associated with no or very mild clinical symptoms and lung lesions (Vicca et al., 2003; Meyns et al., 2006).

Previous studies showed that *M. hyopneumoniae* isolates originating from the same batch of slaughter pigs were very similar at genomic level, whereas isolates originating from different batches showed major diversity at genomic (Stakenborg et al., 2005) and also at proteomic level (Calus et al., 2007). Since infected pigs are commonly transferred from one herd to another, it can be expected that different *M. hyopneumoniae* isolates are circulating within the same herd. This would imply that under field conditions, pigs may become infected with different *M. hyopneumoniae* isolates before they reach slaughter age. The effect of multiple infections with different *M. hyopneumoniae* isolates on the severity of the disease is, however, not known.

The currently available inactivated whole-cell vaccines do not offer complete protection against lung lesions, they do not prevent colonization (Thacker et al., 1999) and they are not able to significantly reduce the transmission of *M. hyopneumoniae* (Meyns et al., 2006). Consequently, they are not suitable to eliminate *M. hyopneumoniae* from infected pig herds neither to end up in a sustainable control of the disease. Attenuated vaccines might be a good alternative. Indeed, for other *Mycoplasma sp.* such as *M. synoviae* in chickens, live
attenuated, temperature sensitive vaccines have been used successfully to control virulent infection (synovitis and respiratory disease) in commercial chicken flocks (Jones et al., 2006).

For *M. hyopneumoniae* it is not known if infection with low virulent isolates may induce protection against highly virulent isolates.

The present study aimed to investigate the effect of infection of pigs with low virulent isolates of *M. hyopneumoniae* on a subsequent challenge infection with a highly virulent isolate.
2. Materials and methods

Animals

Fifty-five, 3 week-old, cross-bred (Rattlerow Seghers®, Buggenhout, Belgium) piglets were weaned and moved to the animal facilities of the Faculty of Veterinary Medicine, Ghent University, Belgium. The piglets were obtained from a herd that was free of *M. hyopneumoniae* and PRRSV. The herd had been monitored repeatedly during the last 8 years by means of clinical, pathological and serological parameters for *M. hyopneumoniae* and PRRSV in sows and pigs of different age categories, and none of the results showed evidence for presence of these pathogens. The piglets were acclimatized during one week, and during the entire trial, they were fed a commercial feed with no antimicrobials.

Experimental design and *M. hyopneumoniae* isolates

After acclimatization, when the pigs were 28 days old (D0), they were randomly allocated to 6 different groups (Table 1). Each group was housed in a different compartment that was equipped with absolute filters (HEPA U15) to avoid spread of the pathogen between groups.

Two different low virulent isolates namely F1 (LV1) and F13 (LV2), and one highly virulent isolate namely F7 (HV) were used for the inoculations. The virulence of these isolates had been tested in previous experimental infection models (Vicca et al., 2003). At D0, groups LV1-HV and LV1 were inoculated with LV1, groups LV2-HV and LV2 with LV2, and group HV with sterile culture medium. Four weeks later (D28), the pigs of these different groups were either intratracheally inoculated with the highly virulent isolate (groups LV1-HV, LV2-HV, HV) or with sterile culture medium (groups LV1 and LV2). A negative control group consisted of pigs inoculated with sterile culture medium on D0 and D28. For the inoculations, the pigs were anaesthetized with 0.22 ml/kg of a mixture of Xyl-M® 2% (Intervet) and Zoletil
100® (Virbac). Depending on the group, they were inoculated intratracheally with either 7 ml of 10^7 color changing units (CCU) per ml of a low or highly virulent *M. hyopneumoniae* isolate, or with 7 ml of sterile culture medium.

All animals were euthanized and necropsied at 28 days after the second inoculation (D56). Therefore, deep anaesthesia was applied by administrating intramuscularly 0.3 ml/kg of a mixture of XylM® 2% and Zoletil 100®, followed by exsanguination.

Table 1: The different groups used to study the effect of infection with low virulent *M. hyopneumoniae* isolates (LV1 and LV2) on subsequent challenge infection with a highly virulent (HV) isolate. The first inoculation took place when the pigs were 4 weeks old (D0) and the second inoculation 4 weeks later (D28). All pigs were euthanized at 4 weeks after the second inoculation (D56).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Inoculation at D0 with</th>
<th>Inoculation at D28 with</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV1</td>
<td>10</td>
<td>LV1</td>
<td>sterile medium</td>
</tr>
<tr>
<td>LV2</td>
<td>10</td>
<td>LV2</td>
<td>sterile medium</td>
</tr>
<tr>
<td>LV1-HV</td>
<td>10</td>
<td>LV1</td>
<td>HV</td>
</tr>
<tr>
<td>LV2-HV</td>
<td>10</td>
<td>LV2</td>
<td>HV</td>
</tr>
<tr>
<td>HV</td>
<td>10</td>
<td>sterile medium</td>
<td>HV</td>
</tr>
<tr>
<td>control</td>
<td>5</td>
<td>sterile medium</td>
<td>sterile medium</td>
</tr>
</tbody>
</table>

*Clinical parameters*

The piglets were observed daily for a minimum of 15 minutes throughout the entire study period (D0-D56). The severity of coughing was graded in a blinded manner by means of a respiratory disease score (RDS) (Halbur et al., 1996). The RDS could range from 0 to 6: 0 (no
coughing), 1 (mild coughing after an encouraged move), 2 (mild coughing while being in rest), 3 (moderate coughing after encouraged move), 4 (moderate coughing while being in rest), 5 (severe coughing after encouraged move), 6 (severe coughing while being in rest). The daily RDS values were summed and averaged for the two periods namely D0-D28 and D28-D56.

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

After necropsy, the lungs were removed and a digital picture was taken from all the lungs for further reference. The macroscopic pneumonia lesions were quantified in a blinded manner using the lung lesion score as described by Hannan et al. (1982). Values could range theoretically from 0 (no lesions) to 35 (entire lung affected).

Lung tissue samples for histopathological examination and IF testing were taken from each apical, cardial and diaphragmatic lobe from each pig. In case lung lesions were present, tissue samples for IF testing were collected from the edge of a lesion.

The tissue samples for histopathological examination were fixed in 10% neutral formalin, processed and embedded in paraffin. One slide was made per lobe sample. Peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation consistent with *M. hyopneumoniae* lesions were scored (0-5) using light microscopy as described previously (Vicca et al., 2003). Scores 1 (limited cellular infiltrates- macrophages and lymphocyte-around bronchioles; with airways and alveolar spaces free of cellular exudates), and 2 (moderate infiltrates with mild diffuse cellular exudates into airways) were classified as lesions not related to *Mycoplasma* infections. Scores 3, 4, 5 were considered to be associated with *Mycoplasma* infection (mild, moderate or severe- broncho-interstitial (cuffing)
pneumonia, surrounding bronchioles but extending to the interstitium, with lymphofollicular infiltration and mixed inflammatory cell exudates) (Morris et al, 1995). One measurement per lobe and three lobes per pig were investigated. The average score per inoculation group was calculated.

IF testing for *M. hyopneumoniae* was done using the assay developed by Kobisch (Kobisch et al., 1978), and the result was expressed semi-quantitatively using scores ranging from 0 to 3: 0 (no IF), 1 (limited IF), 2 (moderate IF), and 3 (intense IF) (Vicca et al., 2003)

**Serology**

Blood samples were taken from all pigs at D0, D14, D28, D42 and D56 and tested for the presence of antibodies against *M. hyopneumoniae* using the DAKO® Mh ELISA (Dako Cytomation, Denmark) (Feld et al., 1992). Sera with optical density (OD)-values < 50% of the OD<sub>buffer-control</sub> were considered positive, OD-values > 50% of the OD<sub>buffer-control</sub> were classified as negative. Intermediate OD-values were considered doubtful and classified as negative.

**BAL fluid and nPCR**

The lungs were removed during necropsy, and bronchoalveolar lavage (BAL) fluid was collected from the left part of the lungs before taking the samples for histopathology and IF. A total of 50 ml of Friis fluid was slowly infused in the lung and aspirated back into the syringe. The recovered fluid was aliquoted, immediately cooled at 4°C, and subsequently stored at -70°C until analysis.

DNA was extracted from BAL fluid with the QIAGEN protocol for purification of total DNA from animal blood and tissues (*QIAGEN, DNeasy Blood & Tissue Kit, Belgium*). For
detection of *M. hyopneumoniae* DNA, a nPCR-test based on the R₁ repeat of P97 was used enabling discrimination between the different *M. hyopneumoniae* isolates used for infection (Gebruers et al., 2008).

**Statistical analysis**

The RDS scores, macroscopic and histopathological lung lesions, percentage of air in the lungs, IF testing and serology were evaluated by using one-way analysis of variance (ANOVA). Levene’s test was used to assess the homogeneity of the variances between the different groups. For each parameter, pair-wise comparisons between groups were made using Bonferoni’s test. RDS scores during D0-D28 and D28-D56 were compared within each group using paired t-tests. Statistical results were considered significant when P-values were ≤0.05. The statistical package SPSS 15.0 for Windows (SPSS 15, SPSS Inc. Illinois, USA) was used to analyse the data.
3. Results

Clinical parameters

Three out of the 55 piglets died during the study: one from the LV1-HV group (at D14), one from the LV2 group (at D18) and one from the control group (at D18). Necropsies were performed to establish the causes of death. The piglet from the LV1-HV group died due to a congenital stenosis of the rectum, the two other piglets died due to meningitis caused by Streptococcus suis serotype 2 infection.

During D0-D28, coughing was absent in the groups that were inoculated with sterile culture medium (group HV, negative control group), and was very mild in the 4 other groups that were infected with a low virulent isolate (Figure 1). Very mild coughing started first in the LV2 group between D7-D10.

During D28-D56, coughing was present in all groups, except for the control group. The RDS values were significantly higher during D28-D56 compared to D0-28 in all infected groups (P<0.05). The highest average RDS values were observed in groups LV1-HV (1.48) and LV2-HV (1.49), the lowest in groups LV1 (0.50) and LV2 (0.64). Intermediate RDS values (0.79) were observed in group HV (Figure 1). The average RDS value was significantly lower in the LV1 group compared to the other infected groups (P<0.05). During the period D28-D56, the severity of coughing increased towards the end of the study.

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

Macroscopic lung lesions were not observed in the negative control group. Only numerical differences (P>0.05) were found between the different infected groups (Table 2). The highest values for macroscopic lesions were observed in group LV1-HV (4.14), HV (3.49) and LV2-HV (2.53), the lowest in groups LV1 (1.92) and LV2 (2.17).
The mean scores of the histopathological lesions in the different groups were 2.27 (LV1), 2.67 (LV2), 3.44 (LV1-HV), 3.17 (LV2-HV), and 3.10 (HV). The mean scores of LV1-HV and LV2-HV were significantly different from those of the LV1 group (P<0.05). The mean score of the control group (0.42) was significantly lower than the mean scores of all other groups (P<0.05) (Table 2). In pigs of the LV1 and LV2 groups, lymphohistiocytic infiltration was predominantly seen in the pulmonary interstitial space, whereas in pigs of the HV group it mainly surrounded the airways. The infiltration in pigs of groups LV1-HV and LV2-HV was present in the pulmonary interstitial space as well as around the airways.

All pigs were positive for IF staining, except for those of the negative control group. The highest mean IF scores were present in the LV1-HV (1.92) and LV2-HV (1.97) groups, but there were no significant differences with the mean scores of the other infected groups (LV1 1.53; LV2 1.48; HV 1.57).
Table 2: Results (average ± SD) of macroscopic and histopathological (lymphohistiocytic infiltration) lung lesion scores and immunofluorescence (IF) scores in the different groups (LV low virulent; HV highly virulent)

<table>
<thead>
<tr>
<th>Group</th>
<th>Macroscopic lung lesions$^1$</th>
<th>Histopathological lung lesions$^1$</th>
<th>IF$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV1</td>
<td>1.92$^a$ ± 1.6</td>
<td>2.27$^b$ ± 0.5</td>
<td>1.53$^a$ ± 0.7</td>
</tr>
<tr>
<td>LV2</td>
<td>2.17$^a$ ± 1.8</td>
<td>2.67$^{ab}$ ± 0.7</td>
<td>1.48$^a$ ± 0.6</td>
</tr>
<tr>
<td>LV1+HV</td>
<td>4.14$^a$ ± 2.3</td>
<td>3.44$^a$ ± 0.7</td>
<td>1.92$^a$ ± 0.4</td>
</tr>
<tr>
<td>LV2+HV</td>
<td>2.53$^a$ ± 2.1</td>
<td>3.17$^a$ ± 0.5</td>
<td>1.97$^a$ ± 0.3</td>
</tr>
<tr>
<td>HV</td>
<td>3.49$^a$ ± 3.3</td>
<td>3.10$^{ab}$ ± 0.7</td>
<td>1.57$^a$ ± 0.5</td>
</tr>
<tr>
<td>control</td>
<td>0.00$^b$ ± 0.0</td>
<td>0.42$^c$ ± 0.2</td>
<td>0.00$^b$ ± 0.0</td>
</tr>
</tbody>
</table>

$^1$ Values with different superscripts within a column are significantly different (P<0.05)

**Serology**

In none of the piglets antibodies to *M. hyopneumoniae* were detected at D0, and the pigs of the negative control group remained negative throughout the entire study (Table 3). The percentage of serologically positive pigs 4 weeks after infection (D28) was 0% for the LV1 and LV1-HV groups, and 33% and 30% for the LV2 and LV2-HV groups, respectively. The percentage of serologically positive pigs at D56, namely 8 weeks after infection with a low virulent isolate, increased to 30% (LV1) and 89% (LV2). Ninety (90) percent of the pigs of the HV group were serologically positive 4 weeks after infection (D56). In almost all pigs in the groups with the combined infections, antibodies to *M. hyopneumoniae* were detected at D56.
Table 3: Percentage of seropositive pigs (average OD-values) in the different groups at different time-points during the experimental study (D0-D56) (LV low virulent; HV highly virulent)

<table>
<thead>
<tr>
<th></th>
<th>D0</th>
<th>D14</th>
<th>D28</th>
<th>D42</th>
<th>D56(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV1</td>
<td>0 (101)</td>
<td>0 (85.7)</td>
<td>0 (74.9)</td>
<td>10 (78.3)</td>
<td>30 (64.3)(^a)</td>
</tr>
<tr>
<td>LV2</td>
<td>0 (89.1)</td>
<td>0 (70)</td>
<td>33 (64.9)</td>
<td>78 (34.8)</td>
<td>89 (19.6)(^b)</td>
</tr>
<tr>
<td>LV1-HV</td>
<td>0 (92.2)</td>
<td>0 (85.6)</td>
<td>0 (70.9)</td>
<td>67 (36.1)</td>
<td>89 (18.1)(^b)</td>
</tr>
<tr>
<td>LV2-HV</td>
<td>0 (94.4)</td>
<td>14 (71.4)</td>
<td>30 (55.1)</td>
<td>80 (31.9)</td>
<td>100 (14.4)(^b)</td>
</tr>
<tr>
<td>HV</td>
<td>0 (94.4)</td>
<td>0 (82.9)</td>
<td>0 (82.1)</td>
<td>10 (69.8)</td>
<td>90 (35)(^b)</td>
</tr>
<tr>
<td>control</td>
<td>0 (96.2)</td>
<td>0 (85.3)</td>
<td>0 (97.7)</td>
<td>0 (86)</td>
<td>0 (92.7)(^c)</td>
</tr>
</tbody>
</table>

\(^1\)Values with different superscripts at D56 are significantly different (P<0.05)

*nPCR testing on BAL fluid*

All piglets from the negative control group were negative using nPCR testing on BAL fluid, all other pigs were positive. In the LV1-HV group, the low virulent and highly virulent isolate were detected in 7/9 and 9/9 of the pigs, respectively. In the LV2-HV group, the low and highly virulent isolate was detected in 7/10 and 10/10 of the pigs, respectively.
4. Discussion

The present study showed that infection with a low virulent *M. hyopneumoniae* isolate does not protect piglets against subsequent infection 4 weeks later with a highly virulent isolate. It appeared, based on clinical parameters and macroscopic and histopathological lesions, that a previous infection with a low virulent isolate may increase the severity of an infection with a highly virulent isolate.

The two low virulent isolates did only induce very mild clinical symptoms during the first 4 weeks after experimental infection. This confirms the results of previous experimental studies with these isolates (Vicca et al., 2003). However, from 4 until 8 weeks post infection, the pigs of the LV1 and LV2 groups developed more severe clinical symptoms and showed mild to moderate macroscopic and microscopic lung lesions. Consequently, it appears that disease signs develop later after infections with these isolates compared to highly virulent isolates. The findings also suggest that the widely used experimental infection model for *M. hyopneumoniae* namely monitoring of the clinical symptoms during 4 weeks combined with evaluating the lung lesions at 4 weeks after challenge infection is not well suited to assess the full virulence capacity of such isolates. The standard infection model for *M. hyopneumoniae* is based on the observation that lung lesions reach their maximal size 2 to 4 weeks after experimental infection (Zielinski et al., 1992; Kobisch et al., 1993). The effect of the inoculation with sterile isolation medium at 28 days after infection with the low virulent isolate is, however, not known and it can not be excluded that this may have influenced the disease outcome.

The percentage of seropositive pigs infected with the low virulent isolates was significantly higher (P<0.05) at D56 (8 weeks after infection) compared to D28 (4 weeks after infection), but the response in group LV1 was significantly lower than in pigs subsequently infected with the highly virulent isolate. In a study designed to quantify the transmission of *M. hyopneumoniae*.
hyopneumoniae, Meyns et al. (2006) also found that pigs infected with a low virulent isolate had a lower and slower serological response compared to pigs infected with a highly virulent isolate. The precise reason for this is not known, but it is possible that low virulent M. hyopneumoniae isolates multiply slower in the lung tissue after infection, and that as a result of this, they trigger the immune response less effectively. Pigs of the LV1 and LV2 groups had the lowest IF scores, suggesting that indeed less M. hyopneumoniae organisms were present in the lung tissue. Young et al. (2000) also stated that highly virulent isolates had a higher capacity to colonize or attach to the ciliated epithelium.

The results confirm that detecting serum antibodies for M. hyopneumoniae is not a reliable tool to assess the infection status of an individual animal (Fano et al., 2005). Even the present experimental procedure in which pigs were inoculated intratracheally with $7 \times 10^7$ CCU of M. hyopneumoniae did not elicit detectable serum antibodies 8 weeks later in 40% of the animals. It also appeared that the serological response was quite variable between the LV1 and LV2 group. At D56, serum antibodies were detected in 30% and 89% of the pigs inoculated with LV1 and LV2, respectively.

The results of the HV group were very similar to those observed in previous experiments with this isolate (Vicca et al., 2003; 2005), confirming the virulence of the isolate and the reproducibility of the experimental infection model used. There were no indications of a protective immunity induced by infection with the low virulent isolates on subsequent infection with the highly virulent isolate. RDS from D28 to D56, microscopic lung lesions, and the intensity of IF staining in the lungs were significantly or numerically higher for the groups with a combined infection (LV1-HV; LV2-HV) than for groups inoculated with either only a low or highly virulent isolate. This suggests that low virulent isolates, although they do not induce severe clinical symptoms by themselves, may increase the severity of subsequent infection with a highly virulent isolate. The exact mechanisms for this interaction are not
It may be related to a more pronounced inflammatory reaction of the host, as the inflammatory response is known to be involved in the clinical symptoms and lung lesions (Thacker et al., 2006).

In the current experiment, pigs were intratracheally inoculated with high doses of *M. hyopneumoniae*. Under field conditions, infection doses are not known but they are probably lower than the $7 \times 10^7$ CCU used in this study. The higher inoculation dose in our experiment may result in a faster and a higher level of colonization at the surface of the airways, compared to field infections. This agrees with the observation of a faster induction of seroconversion in experimentally inoculated animals compared to contact infected animals (Feld et al., 1992; Fano et al., 2005). On the other hand, the housing conditions, management practices and air quality in the experimental setting were better than those present under field conditions. Also, infections with pathogens like PRRSV were not present in our experimental animals. These infections may influence the clinical outcome of *M. hyopneumoniae* infections (Thacker et al., 1999). Although extrapolation of results obtained in experimentally infected pigs to the field situation should be done with caution, the infection model used allows studying the effects of infections with *M. hyopneumoniae* of different virulence in a standardized and reproducible way.

In conclusion, the present study demonstrates that pigs inoculated with low virulent isolates of *M. hyopneumoniae* isolates are not protected against a subsequent infection with a highly virulent isolate 4 weeks later and may even develop more severe disease signs. This indicates that subsequent infections with different *M. hyopneumoniae* isolates may lead to more severe clinical disease in a pig herd. Further research is necessary to assess the precise mechanisms influencing the interactions between highly and low virulent isolates, and to investigate the importance of the present findings under field conditions.
Acknowledgements

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CHAPTER 4.

GENERAL DISCUSSION
GENERAL DISCUSSION

*M. hyopneumoniae* is the causal agent of enzootic pneumonia in pigs and represents a costly problem for the pig industry worldwide. The clinical manifestation of the disease is the result of a combination of farm management practices, environmental factors, secondary infections and the virulence of the *M. hyopneumoniae* strains present in a herd (Vicca et al., 2003). Notwithstanding the efforts and control strategies adopted to rear high health pig herds, *M. hyopneumoniae* continues to be a widely spread organism in pig populations, which renders eradication of this respiratory disease a very difficult and frustrating task. The present thesis aimed to obtain a better epidemiological insight into early infections with *M. hyopneumoniae* in herds with respiratory problems and the transmission in nursery pigs under field conditions. Furthermore, the protection against experimental infection with different strains of *M. hyopneumoniae* was investigated. On the one hand, the potential protection offered by a low virulent strain was assessed. On the other hand, the efficacy of a currently existing vaccine against infections with a low and a highly virulent strain was investigated. The present chapter will provide a general discussion of the findings obtained in the different studies.

**Epidemiology of *M. hyopneumoniae* infections in young pigs from herds with respiratory problems**

*M. hyopneumoniae* infections starting early in the production system play a critical role in later development of respiratory disease in a pig herd (Fano et al., 2007). Although clinical symptoms are not common before 6 weeks of age (Ross et al., 1999), it is known that the lower and upper respiratory tract of piglets can already be colonized by *M. hyopneumoniae* before weaning (Sibila et al., 2007; Moorkamp et al., 2009). These authors suggested that prevalence at weaning can be used to predict the clinical course and severity of the disease later on during the fattening period. Thus, to have a better understanding of the infection
dynamics of this pathogen, it is important to assess the infection level at an early age. In our first study (3.1), the infection level was estimated in piglets of 3 weeks of age in different types of pig herds with respiratory problems in grow-finish pigs located in nine different European countries. Of the total 52 pig herds included in this study, 28.6% were farrow-to-finish herds, 26.5% and 24.5% were sow herds from which the piglets were moved to another farm after weaning or at 20-25 kg, respectively, and the remainder of the herds (20.4%) raised a part of the fattening pigs on the same site.

The total average percentage of positive piglets was 10.7%. The results also showed that in two thirds of the studied herds, there was evidence of *M. hyopneumoniae* infection in 3-week old piglets. In herds vaccinating against *M. hyopneumoniae* 8.4% (95% CI 7.6-9.1) were infected, whereas only 2.6% (95% CI 1.8-3.4) were infected in the herds not vaccinating against *M. hyopneumoniae*. In most of the positive herds, the infection rate varied between 1 and 10%, whereas in some others more than 30% of the piglets were already positive.

The prevalence of *M. hyopneumoniae* in suckling piglets under field conditions has been documented in other studies, but these studies were conducted in a limited number of herds and in a particular geographic region (Sibila et al., 2007; Moorkamp et al., 2009; Nathues et al., 2010). Yet, the average detection rate of 10.7% in suckling pigs estimated by our study fits within the range of observations presented by other authors, considering that many of them also included herds with a history of endemic respiratory disease. For instance, Calsamiglia and Pijoan (2000) reported between 7.7% - 9.6% of pigs infected by 17 days of age. Sibila et al. (2007) showed that the percentage of suckling pigs with *M. hyopneumoniae* detection in nasal swabs was 1.5% - 3.8%. Nathues et al. (2010) observed an infection rate of 2.0%, while another study reported a higher prevalence of 12.3% in piglets of the same age range (Moorkamp et al., 2009). Only one author reported a very wide range of infection (2.5 - 51.8%) in piglets from different batches all belonging to a multi-site farm, one day before
weaning (Fano et al., 2006). However, such large variability could be due to the small number of pigs sampled in each batch. Our study was the first to investigate the occurrence of this pathogen in different countries in Europe and the results showed that in almost all countries and type of herds, early *M. hyopneumoniae* infections are important.

A limited number of herds were included in the study; thus, the interpretation of these results should be confined to herds encompassing the selection criteria, instead of being extrapolated to the total population of pig herds within Europe. Generalization of these data might either underestimate or overestimate the true prevalence of *M. hyopneumoniae* in suckling piglets under field conditions.

It has been found that the prevalence of *M. hyopneumoniae* in young piglets may be associated with different factors such as one-site and two-site production, the presence of other respiratory pathogens in the herd (PRRSV, swine influenza virus, *Pasteurella multocida*, *Haemophilus parasuis*, *Mycoplasma hyorhinis*, *Actinobacillus pleuropneumoniae* and *Streptococcus suis*) and inappropriate acclimatization of replacement gilts (Moorkamp et al., 2009). In the final multivariable model of our study, it was shown that early *M. hyopneumoniae* infection was correlated to herds practicing sow vaccination against swine influenza virus (SIV). The exact reason for the higher risk of infection in herds vaccinating their sows against SIV is not known. A possible explanation may be that pig herds with respiratory symptoms are more likely to vaccinate their sows against SIV, overlooking the possible influence of other respiratory pathogens such as *M. hyopneumoniae*. As most of the respiratory problems are due to multiple infections and also to non-infectious factors (Thacker et al., 2002), it is possible that in these herds, *M. hyopneumoniae* and/or other pathogens are involved in respiratory problems in addition to SIV.

Apart from the sows playing a key role in the infection of their offspring, there are other factors that may influence infection at a young age. For instance, piglets from different sows
are often grouped together shortly after birth and later at weaning. Separation from the dam, change of feed, housing, as well as the social stress inflicted by the effect of mixing, hierarchy competition and dominance status during this period may suppress their immune function and make them more vulnerable to infection (De Groot et al., 2001). As a consequence, *M. hyopneumoniae* organisms continue to spread horizontally between pen mates in the nursery unit (Sibila et al., 2009). Moreover, airborne transmission can also occur, leading to transmission of *M. hyopneumoniae* between pigs of different units within a herd. The detection rate of *M. hyopneumoniae* in the piglets was not significantly different between herds practicing vaccination and those not practicing vaccination. The absence of a difference in the present study can be due to differences in vaccination strategies employed by the herds (e.g. type of vaccine, timing of vaccination, etc). Piglet vaccination constitutes one of the main strategies currently used for control of *M. hyopneumoniae* infection. Therefore, it is important to assess whether the vaccination of weaned piglets influences the extent of *M. hyopneumoniae* transmission during the nursery period.

**Transmission of *M. hyopneumoniae* in vaccinated and non-vaccinated nursery pigs**

Our second study (3.2) aimed to quantify *M. hyopneumoniae* transmission in nursery pigs under field conditions, and to assess the effect of vaccination on transmission. Broncho-alveolar lavage fluid was collected at weaning and at the end of the nursery period to assess the presence of *M. hyopneumoniae* by nPCR and to calculate the reproduction ratio ($R_n$). The average $R_n$-values obtained were 0.56 and 0.71 for the non vaccinated and the vaccinated group, respectively, indicating that vaccination alone was not able to significantly reduce the transmission of this pathogen in nursery pigs in the present herd.

An earlier trial under experimental conditions (Meyns et al., 2006) showed similar findings and demonstrated that vaccination with another conventional vaccine could not prevent
infection nor transmission of this organism. The average \( R_n \) values obtained in our study were lower than those obtained under experimental conditions (Meyns et al., 2004, 2006), which could be influenced, among other factors, by the infection dose. The infection dose in the present study is not known, but it is expected that the dose used in the previous experimental trials (Meyns et al., 2004; 2006) was higher than the infection dose under field conditions at weaning. A lower infection dose may result in a lower number of \( M. \) hyopneumoniae organisms in the respiratory tract, a lower transmission rate, and consequently, a lower \( R_n \) value.

Despite the lack of significance in the difference of the transmission rate between vaccinated and non vaccinated animals, some observations suggest that the load of \( M. \) hyopneumoniae in the lungs of infected animals may be influenced by vaccination. For instance, Meyns et al. (2006) used an IF-staining method to measure in a semi-quantitative way the number of \( M. \) hyopneumoniae bacteria in the lung tissue of pigs. They found that the lung samples of vaccinated animals had a lower average IF score than the lungs of non vaccinated ones, possibly also resulting in lower excretion.

The virulence of the \( M. \) hyopneumoniae strain might influence the number of organisms present in the respiratory tract of an infected pig. Although Meyns et al. (2004) did not find statistically significant differences between transmission rate of a highly and a low virulent strain, it was shown that with the same infection dose, the lung tissue of pigs infected with a highly virulent strain displayed a more intense IF-staining than the lung tissue of pigs infected with a low virulent strain (Vicca et al., 2003; Meyns et al., 2004). In addition, the titer of \( M. \) hyopneumoniae was significantly higher in the BAL fluids of the pigs infected with a highly virulent strain at 10 and 15 DPI compared with the titer in BAL fluid of pigs infected with the low virulent strain (Meyns et al., 2007). It was suggested that, among other factors, the faster \textit{in vivo} multiplying capacity of the highly virulent strain could explain the higher titers found.
in the lungs. The virulence of the *M. hyopneumoniae* strain in our field study was not known, and also, it was not known whether the pigs were infected with only one or with different *M. hyopneumoniae* strains. Previous research has shown that diversity among *M. hyopneumoniae* strains circulating in pig herds may occur (Vicca et al., 2003; Calus et al., 2007). Further research however is necessary to assess the importance of this diversity from a clinical and epidemiological point of view, and ultimately, for the implementation of optimal control strategies.

In our study, piglets were born from sows vaccinated against *M. hyopneumoniae* at 5 and 3 weeks prior to farrowing. The effect of sow vaccination has been documented by several studies (Ruiz et al., 2003, Kristensen et al., 2004, Beilage et al., 2005, Martelli et al., 2006, Sibila et al., 2008), and it is known to enhance the levels of maternally derived antibodies during the first weeks of life of the offspring. At the same time, in some studies, it seems to be able to reduce prevalence of *M. hyopneumoniae* in piglets at weaning at the herd level (Ruiz et al., 2003; Beilage et al., 2005). Thacker et al. (2000) however observed only limited to no effect on respiratory tract colonisation by this pathogen.

It is not clear whether high levels of maternally derived antibodies may interfere with early (one-shot) vaccination (Beilage et al., 2005). According to a study by Martelli et al. (2006), sow vaccination did not have an effect on the serological response of vaccinated piglets. It is difficult, therefore, to say whether the higher level of antibodies in the vaccinated than in the non-vaccinated group at the start of this study may have interfered with the efficacy of vaccination at 3 weeks of age, and possibly influenced the transmission ratio among piglets. More research on the influence of maternally derived antibodies on the immunization of piglets, and the best timing for vaccination, is necessary.

The $R_n$ calculations in this study were based on the number of pigs infected at the end of the nursery period. To measure the infectious state of the animals, nPCR was performed on BAL
fluid obtained from live anesthesized animals. Since *M. hyopneumoniae* attaches to the ciliated epithelium of the respiratory tract, the best samples to detect this organism by nPCR are tracheo-bronchial swabs or BAL fluid (Marois et al., 2008; Fablet et al., 2010). BAL fluid collection can be time and labour-expensive, but the high specificity and sensitivity of nPCR on this type of sample offers several advantages over other diagnostic methods. Its sensitivity enables the detection of \(5 \times 10^6\) ng *M. hyopneumoniae* DNA or as few as 4 organisms / µl reaction mixture (Gebruers et al., 2008). For practical reasons, other studies have used nPCR on nasal swabs to determine prevalence in young piglets (Sibila et al., 2007). However, the number of organisms in the upper respiratory tract is lower and organisms are shed intermittently (Kurth et al., 2002; Ruiz et al., 2002; Pieters et al., 2009). In addition, inhibiting factors such as mucin can also interfere in the DNA extraction and affect the process of amplification (Vranckx K. 2010, personal communication). Nonetheless, nPCR performed on nasal swabs is considered to be appropriate to assess the presence of *M. hyopneumoniae* at herd level, but not to assess the infection at the animal level (Otagiri et al., 2005; Sibila et al., 2009).

Until recently, no accurate diagnostic tools were available for quantifying the bacterial load of this organism in the BAL fluid from live naturally infected animals. However, new methods have recently been developed to increase accuracy in the measurements. The best quantitative technique available nowadays is the real-time PCR assay for *M. hyopneumoniae* (Marois et al., 2010; Fablet et al., 2010). Real-time PCR is an accurate, fast and easy to perform technique that offers new possibilities for transmission studies, taking epidemiological research from qualitative identification to precise quantification of this organism. Although this tool was still under development during the performance of our study, it should certainly be considered in future studies investigating the effect of vaccination on the possible reduction of the load of this microorganism in the respiratory tract of infected pigs. The
quantification of the number of organisms being shed by an animal per unit of time would provide highly valuable information in *M. hyopneumoniae* research and could reveal new insights in the transmission of this pathogen.

It is important to point out that the $R_n$-values in this study correspond only to the nursery population. An $R_n$-value lower than one would mean lack of transmission only at that stage, but not in the whole pig population, since infection could be already established at an earlier age (chapter 3.1) or could occur at a later stage. Thus, eradication of *M. hyopneumoniae* will only be achieved when appropriate control measures will lower the average $R_n$-value to less than one for all circulating *M. hyopneumoniae* strains, at all stages in the pig population. To achieve such results, a vaccine would either need to decrease the infectivity of animals that spread the pathogen and/or induce better resistance in animals that are still susceptible to infection. So, additional control measures and/or more efficient vaccines will be needed to achieve this goal.

Infection pattern of *M. hyopneumoniae* strains of different virulence in vaccinated and non-vaccinated pigs

From our study (3.2) and previous experimental trials (Meyns et al., 2006; Pieters et al., 2010), it is clear that the current commercial vaccines are not able to stop transmission. However, little is known about the effect of vaccination against strains of different virulence. Strait et al. (2008) suggested that differences in virulence may affect the ability of current vaccines to protect against different *M. hyopneumoniae* field isolates. Our third study (3.3) aimed to investigate the infection pattern and the lung lesion development caused by a low and a highly virulent strain, and to examine the efficacy of vaccination against each one of them.
This study confirmed the difference in virulence between these two strains both at 4 weeks PI and at 8 weeks PI. In the commonly accepted standard infection model (Whittlestone et al., 1972; Kobisch et al., 1993) used in previous experiments, clinical signs and lung lesions are assessed up to 4 weeks PI. Our study was the first experimental trial to examine the pattern of infection of both strains over a period of 8 weeks PI.

It was demonstrated that, contrary to the highly virulent strain, the low virulent strain requires more than 4 weeks PI to reach maximum symptoms. Apart from developing milder symptoms, the onset of disease in pigs infected with the low virulent strain only started at about 4 weeks PI, whereas at this point in time symptoms were already at their maximum in pigs infected with the highly virulent strain. This is a clear indication that the standard infection model is not entirely suitable for the evaluation of the course of disease caused by low virulent strains. For these *M. hyopneumoniae* strains, it is necessary to observe the pigs for a longer period than the traditional 4 weeks period. Further research is necessary to investigate why the infection and disease course is different between low and highly virulent strains.

In addition to the differences in virulence and effect of time on the infection course, the efficacy of vaccination against highly and low virulent strains was also evaluated. It was observed that vaccination delayed the clinical onset of disease and significantly reduced its severity, both in pigs infected with the highly virulent strain and in those infected with the low virulent strain. Disease reduction by vaccination, however, was more prominent in pigs challenged with the highly virulent strain than in pigs challenged with the low virulent strain. Part of the different effects might be explained by the fact that significant vaccination efficacy is more visible when clinical symptoms and lung lesions are more severe, leaving more room for improvement by vaccination. Another possibility is that vaccination may affect differently the multiplication rate of *M. hyopneumoniae* in the lungs for the highly and low virulent
strain. Vaccination significantly reduced IF scores in the pigs infected with the highly virulent strain, but not in pigs infected with the low virulent strain. This might suggest that vaccination inhibits the multiplication of the highly virulent strain to a higher extent compared to the low virulent one. It would be interesting to quantify more accurately the effect of vaccination on the load of organisms in the lungs, for instance by using real-time PCR.

To better understand the effect of vaccination on multiplication of different strains in the respiratory tract, more information is also required on the immunological response of the host. The existing reports on immunity induced by vaccination suggest that both cellular and humoral responses are involved in protection (Chen et al., 2008; Strait et al., 2008). Strains with different virulence might trigger mechanisms of the immune response to a different extent (Meyns et al., 2007). In fact, a higher proportion of neutrophils in BAL fluid and higher level of TNF-α have been reported for non vaccinated pigs infected with the highly virulent strain, indicating that the inflammatory cell response is more pronounced in case of infections with the highly virulent strain (Meyns et al., 2007). Whether this also occurs in vaccinated pigs and whether this affects vaccine efficacy may be the subject of further studies.

Since infected pigs are often purchased and transferred from one herd to another, it can be expected that different strains may be circulating under field conditions (Mayor et al., 2007). A high heterogeneity at the genomic and proteomic level has been demonstrated among different M. hyopneumoniae strains (Artiushin and Minion, 1996; Kokotovic et al., 1999; Stakenborg et al., 2005; Calus et al., 2007). Some of these variations among field strains could be held responsible for the variable outcome of vaccination strategies in different herds (Maes et al., 1999). Also, a high variation in virulence exists between M. hyopneumoniae strains isolated from different swine herds (Vicca et al., 2003). However, the virulence factors of M. hyopneumoniae have not been identified and the protective antigens remain undefined. The importance of virulence differences under field conditions for protective immunity by
vaccination is unknown. Further research is necessary to investigate the role of diversity between strains under field conditions and cross-protection between a commercial vaccine strain and the most prevalent strains in a herd. A possible future for the development of alternative vaccines may lie in the identification of new, preferably protective, antigens from different strains. These antigens could then be included in the vaccine, allowing a broader antigenic coverage against different *M. hyopneumoniae* strains.

Adjuvants may also play an important role in the enhancement of the immunogenicity of an antigen. In the case of commercial vaccines against *M. hyopneumoniae*, most of the formulations contain oil-based adjuvants (usually in the form of emulsions), giving a more prolonged release of the antigen and stimulation of the immune system (Groth et al., 2001). An adjuvant consisting of a mixture of amphigen and lecithin was present in the vaccine formulation used in our study (3.3). Although the currently used bacterin formulations are able to reduce lung lesions and improve performance parameters of pigs affected by *M. hyopneumoniae*, the advantages of different adjuvants are still under debate. More studies could elucidate the protective effect of different vaccine formulations against infection with different *M. hyopneumoniae* strains.

Combined infections with different *M. hyopneumoniae* strains

The effect of combined infections with different *M. hyopneumoniae* strains had previously been reported in only one study (Strait et al., 2008). These authors investigated the effect of vaccination on the infection with two contemporary pathogenic isolates of *M. hyopneumoniae*. However, the possible protective effect offered by infection with one strain against subsequent infection with another strain was not investigated. Therefore, in chapter
3.4, we investigated the effect of infection of pigs with two different low virulent strains (F1 and F13) on a subsequent challenge infection with a highly virulent (F7) strain.

The results clearly showed that the low virulent strains were not able to induce protection against the highly virulent strain. On the contrary, the severity of the clinical parameters, macroscopic, histopathological lesions and IF scores was exacerbated in pigs that had been first infected with a low virulent strain. This suggests that although low virulent strains do not induce severe clinical symptoms by themselves, they may play an important role in increasing the severity of subsequent infection with a highly virulent isolate. These results were surprising, since we expected the low virulent strain to confer at least a certain degree of protection against the highly virulent strain. Contrary to our study, Kobisch et al. (1993) found in a limited study that resistance to pneumonia was observed in pigs after a booster infection with the same isolate of *M. hyopneumoniae*. The exact reason for the lack of protection of the low strains against the highly virulent strain in our study is not known, but it might be that the host immune system responds with a more pronounced inflammatory reaction when subsequently exposed to more than one strain. The route of inoculation (endotracheal), being a rather invasive procedure, could also have contributed a more pronounced inflammatory response. For instance, it has been demonstrated that animals receiving particles through inhalation showed a decreased pulmonary response, in both severity and persistence, when compared with those receiving particles through instillation (Osier et al., 1997). The differences in pulmonary response when using alternative routes of infection may be caused by differences in dose rate, particle distribution, or altered clearance.

Compared to natural infection (under field conditions), the inoculation dose with each one of the strains is likely to be higher under the experimental conditions. This might result in a faster and a higher level of colonization of the ciliated surface of the respiratory tract by *M. hyopneumoniae* organisms, triggering more pronounced symptoms and lesions.
Another possibility could be that the low virulent strains require more than 4 weeks to induce protection. Therefore, the time between the first and the second challenge could have been insufficient to trigger a full protective immune reaction in the host. A short-time interval between subsequent exposures of different strains may leave a window of susceptibility that can result in super-infection with exacerbated clinical signs and lung lesions. Superinfection is defined as infection with a second strain after the establishment of persistent infection and development of an immunologic response to the first strain (Blackard et al. 2007). The importance of time interval between challenge exposure is not well documented for \textit{M. hyopneumoniae}, but it is a factor to be considered in future experiments. More knowledge about the immunological response in the animal following \textit{M. hyopneumoniae} infection with individual strains, as well as the optimal time between exposures are necessary for a better understanding of the interaction between strains of different virulence.

As the results did not give any indications of a protective immunity induced by infection with the low virulent strains on subsequent infection with a highly virulent strain, the low virulent strains used in this study should be regarded cautiously as candidates for vaccines. Given the time interval between primary and subsequent exposure, the results also imply that infection with more than one \textit{M. hyopneumoniae} strain may lead to more severe clinical disease in a pig herd.

**General conclusions**

From this thesis, it can be concluded that:

- \textit{M. hyopneumoniae} infections in 3-week old piglets commonly occur in European pig herds suffering from respiratory disease in grow-finishing pigs.
- Vaccination against \textit{M. hyopneumoniae} is not able to significantly reduce the transmission of this pathogen in nursery pigs under field conditions.
• Infection with a low virulent *M. hyopneumoniae* strain requires more than 4 weeks to reach maximum symptoms, indicating that the standard *M. hyopneumoniae* infection model based on a 4-week observation period is not entirely suitable to evaluate low virulent strains.

• A commercial bacterin seems to be more efficacious against infection with the highly virulent *M. hyopneumoniae* strain than with the low virulent one used in this study.

• Previous infection with the low virulent *M. hyopneumoniae* strains does not confer protection against subsequent infection with the highly virulent strain used in this study.
Future perspectives

From the literature review and this discussion, it appears that still many questions on *M. hyopneumoniae* infections remain unanswered. Therefore, further research to answer these questions and to achieve a better and more sustainable control of enzootic pneumonia is necessary.

The prevalence of *M. hyopneumoniae* infections in young piglets at weaning has been addressed, and possible risk factors have been investigated. The sow is known to play an important role in the transmission of this pathogen to her offspring. However, the transmission rate of this pathogen from sows to piglets has not yet been quantified, and the difference between young and older sows in transmission of *M. hyopneumoniae* to the offspring is not known. Also, factors influencing the occurrence and transmission of *M. hyopneumoniae* infections in sows are largely unknown. These may include housing conditions and management practice, parity and vaccination schemes in the herd, amongst others.

No significant difference in transmission rate was observed between piglets vaccinated against *M. hyopneumoniae* and non vaccinated ones. However, it is not known whether vaccination can reduce the load of *M. hyopneumoniae* bacteria in the lungs of infected piglets. Thus, future studies should quantify more accurately *e.g.* by using qPCR, the number of *M. hyopneumoniae* bacteria in the lungs and should investigate whether control measures are able to significantly decrease the infection load in the lungs.

Finally, the interactions between highly and low virulent strains need to be better understood. In our studies, pigs subsequently infected with different strains developed more severe clinical symptoms and lesions. The clinical importance of this finding in pig herds is not known. It is, yet, to be found whether the time interval between challenge exposures plays a
role in the degree of protection conferred. It is also not known whether simultaneous infection with two different strains will lead to more severe disease, and whether both strains will multiply at the same rate in the pig, or whether one strain will dominate the other one. The interactions between *M. hyopneumoniae* and other respiratory pathogens also need to be better understood.

These are important questions as they can help explaining the clinical course of the infection, the persistence of infection in pigs and the chronic nature of the disease in pig herds. Also the effect of vaccination against simultaneous infection with different strains should be investigated, as this may help explaining the variable vaccination results observed in pig herds.
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CHAPTER 4: DISCUSSION


SUMMARY

*M. hyopneumoniae* is the primary agent of enzootic pneumonia, a chronic respiratory disease in pigs that occurs worldwide and causes major economic losses. The clinical course of infections may vary significantly between pig herds depending on herd management strategies, secondary infections and environmental conditions. Also the virulence of the *M. hyopneumoniae* strains may determine the clinical outcome, which renders control of this pathogen a difficult task. Control measures include the optimization of management and housing conditions, the use of strategic medication and/or vaccination. The current control measures are beneficial from an economic point of view, but they do not entail a sustainable solution for the disease.

There is limited information on the occurrence and risk factors involved in early infection and there are still many questions regarding the effect of vaccination on transmission of this pathogen. The limited studies available have mainly focused on experimental conditions and do not necessarily reflect the situation in the field. In addition, information on differences between infection course caused by low and highly virulent field strains and the effect of vaccination on infection with different *M. hyopneumoniae* strains is very scarce. Answers to these questions are necessary for a better understanding of the pathogenicity of this microorganism, and to improve the control of enzootic pneumonia.

The general aim of this study was to assess the importance of early infection and transmission of *M. hyopneumoniae* under field conditions and to investigate protection against different *M. hyopneumoniae* strains.

In the first study (Chapter 3.1), the detection rate of *M. hyopneumoniae* in 3-week-old pigs in different European countries was estimated and possible risk factors were identified. Nasal swabs from suckling pigs in 52 farms were collected for analysis using nested PCR. Potential risk factors for respiratory disease were analysed with a multivariable logistic regression
model. The average percentage of positive piglets was 10.7% (95% confidence interval, CI 7.4-14.2); at least one pig tested positive in 68% of herds. In 32% of the herds, more than 10% of piglets tested positive. Herds that vaccinated sows against swine influenza virus (SIV) had a significantly higher risk of a piglet being positive for *M. hyopneumoniae* (OR 3.12; 95% CI 1.43-6.83). The higher risk in case of SIV vaccination is difficult to explain, but it may be due to the fact that pig herds with respiratory symptoms are more likely to be vaccinated against SIV, overlooking the possible influence of other respiratory pathogens such as *M. hyopneumoniae*. Our results confirmed that *M. hyopneumoniae* is widespread in 3-week-old piglets across different European countries.

Piglet vaccination constitutes one of the main strategies currently used for control of *M. hyopneumoniae* infections. However, it is not known whether vaccination of weaned piglets influences the extent of *M. hyopneumoniae* transmission under field conditions during the nursery period. In Chapter 3.2, a study investigated the effect of vaccination against *Mycoplasma hyopneumoniae* on its transmission in nursery pigs under field conditions. Seventy two pigs were randomly allocated at weaning into vaccinated (V) and non-vaccinated (NV) groups. Animals in the V group were vaccinated at 3 weeks-of-age with a commercial *M. hyopneumoniae* bacterin vaccine. Bronchoalveolar lavage fluid taken at weaning and at the end of the nursery period was assessed for the presence of *M. hyopneumoniae* by nested PCR, and the reproduction ratio of infection (R₀) was calculated. The percentage of positive pigs in the V and NV groups was 14% and 36% at weaning, and 31% and 64% at the end of the nursery period, respectively. The R₀-values for the V and NV groups were 0.71 and 0.56, respectively (*P* > 0.05). The study indicated that vaccination does not significantly reduce the transmission of this respiratory pathogen.

In the third study (Chapter 3.3), the infection pattern and lung lesion development in pigs caused by a low and highly virulent *M. hyopneumoniae* strain at 4 and 8 weeks (w) post
infection (PI) was investigated. The efficacy of a commercial inactivated whole-cell vaccine against infection with each one of these *M. hyopneumoniae* strains was also determined. Ninety piglets free of *M. hyopneumoniae* were selected, and 40 of them were randomly vaccinated during their first week of life. At weaning, all piglets were allocated to 10 different groups and housed in pens with absolute filters. At 4 weeks of age, pigs were inoculated intratracheally with either a highly virulent *M. hyopneumoniae* strain, a low virulent strain or with sterile culture medium. Half of all animals were euthanized at 4w PI, while the remaining half was euthanized at 8w PI. Coughing was assessed daily, and lung lesions, immunofluorescence (IF), bacteriological analysis and nested PCR were assessed after necropsy. It was demonstrated that contrary to the highly virulent strain, the low virulent strain required more than 4 weeks PI (commonly accepted as the standard infection model) to reach maximum clinical symptoms. Vaccination significantly reduced clinical symptoms, macroscopic and microscopic lung lesions in pigs infected with the highly virulent strain. This effect was more pronounced at 4 than at 8 weeks PI. Protective efficacy was also observed in pigs infected with the low virulent strain, but the effect was less pronounced than on the highly virulent strain.

Finally, the effect of an infection with low virulent isolates of *M. hyopneumoniae* (LV1 and LV2) on the subsequent infection with a highly virulent isolate (HV) was evaluated (Chapter 3.4). Fifty-five, 3 week-old piglets free of *M. hyopneumoniae* were randomly allocated to 6 different groups. At 4 weeks of age (D0), groups LV1-HV and LV1 were intratracheally inoculated with LV1, groups LV2-HV and LV2 with LV2, and group HV with sterile culture medium. Four weeks later (D28), the pigs of these different groups were either intratracheally inoculated with the highly virulent isolate (groups LV1-HV, LV2-HV, HV) or with sterile culture medium (groups LV1 and LV2). A negative control group consisted of pigs inoculated with sterile culture medium on D0 and D28. All animals were necropsied at 28 days after the
second inoculation. Clinical symptoms were evaluated daily using a respiratory disease score (RDS). After necropsy, macroscopic and histopathological lung lesions were quantified and immunofluorescence (IF) testing on lung tissue and nested PCR on BAL fluid were performed for the detection of *M. hyopneumoniae*. Disease signs and lung lesions were not observed in pigs of the negative control group. In the other groups, there were no or only very mild clinical symptoms from D0 until D28. A significant increase in the average RDS values was, however, observed during D28-D56, especially in groups LV1-HV (1.48) and LV2-HV (1.49), in group HV (0.79), and to a lesser extent in groups LV1 (0.50) and LV2 (0.65) (P<0.05). The clinical symptoms during D28-D56, the lung lesions and intensity of IF staining were more pronounced in groups LV1-HV, LV2-HV and HV compared to groups LV1 and LV2. All pigs, except those from the negative control group, were positive on IF testing and PCR at D56. The study demonstrated that pigs inoculated with low virulent isolates of *M. hyopneumoniae* are not protected against a subsequent infection with a highly virulent isolate 4 weeks later and may even develop more severe disease signs. This indicated that subsequent infections with different *M. hyopneumoniae* isolates may lead to more severe clinical disease in a pig herd.

From the studies included in this thesis, it was concluded that *M. hyopneumoniae* infections start at an early age and are widely spread in European pig herds. Many questions remain still unanswered and more detailed studies are necessary to assess the possible risk factors associated to these infections.

No significant difference in transmission rate of *M. hyopneumoniae* was observed between piglets vaccinated against *M. hyopneumoniae* and non vaccinated ones. This means that vaccination alone will not be sufficient to significantly decrease *M. hyopneumoniae* infections and that additional control measures will be needed for elimination of the pathogen from pig herds. Future studies should quantify more accurately the number of *M. hyopneumoniae*
bacteria in the lungs and should investigate whether control measures are able to significantly decrease the infection load in the lungs.

The infection pattern and disease course following experimental infection differ between high and low virulent M. hyopneumoniae strains, and also the efficacy of vaccination seems to be determined by the type of strain. A better understanding of the infection pattern and the interactions between highly and low virulent strains is necessary. Pigs subsequently infected with different strains developed more severe clinical symptoms and lesions. However, it is not known whether simultaneous infection with two different strains will lead to more severe disease, and whether both strains will multiply at the same rate in the pig, or whether one strain will dominate the other one. These are important questions as they can help explaining the clinical course of the infection and improve the currently existing control measures against enzootic pneumonia.
SAMENVATTING

*M. hyopneumoniae* is het belangrijkste agens van enzoötische pneumonie, een chronische luchtwegaandoening bij varkens die wereldwijd voorkomt en leidt tot grote economische verliezen. Het klinisch verloop van mycoplasma-infecties kan aanzienlijk verschillen tussen varkensbedrijven afhankelijk van managementstrategieën en omgevingscondities. Ook de virulentie van de *M. hyopneumoniae* stammen kan de klinische uitkomst bepalen, waardoor de controle van deze ziekteverwekker een moeilijke zaak is. Controlemaatregelen omvatten het optimaliseren van de bedrijfsvoering en de leefomstandigheden van de dieren, het gebruik van strategische medicatie en / of vaccinatie. De huidige controlemaatregelen zijn gunstig vanuit economisch oogpunt, maar ze leiden niet tot een duurzame oplossing voor de ziekte.

Er is slechts beperkte informatie beschikbaar over het voorkomen van risicofactoren die betrokken zijn bij vroege infecties en er zijn nog veel vragen over het effect van vaccinatie op de verspreiding van de ziekteverwekker. De beperkte beschikbare studies richten zich voornamelijk op experimentele condities en komen niet noodzakelijk overeen met de situatie in het veld. Bovendien is informatie over de verschillen die er bestaan tussen het verloop van infecties die veroorzaakt worden door laag en door hoog virulente stammen en het effect van vaccinatie op infecties met verschillende *M. hyopneumoniae* stammen, zeer schaars. Antwoorden op deze vragen zijn nodig voor een beter begrip van de pathogeniciteit van dit micro-organisme, en om de bestrijding van enzoötische pneumonie te verbeteren.

Het algemene doel van het onderhavige onderzoek was om het belang van vroege infectie en transmissie van *M. hyopneumoniae* onder veldomstandigheden te beoordelen en om de bescherming tegen verschillende *M. hyopneumoniae* stammen te onderzoeken.

In de eerste studie (hoofdstuk 3.1), werd de prevalentie van *M. hyopneumoniae* bij 3-weken oude varkens in verschillende Europese landen geschat en werden mogelijke risicofactoren
geïdentificeerd. Op 52 bedrijven werden nasale swabs van gespeende biggen genomen voor analyse met behulp van nested PCR. Potentiële risicofactoren voor respiratoire aandoeningen werden geanalyseerd met een multivariabel logistisch regressie model. Het gemiddelde percentage positieve biggen was 10,7% (95% betrouwbaarheidsinterval, CI 7,4-14,2). Op 68% van de bedrijven testte tenminste één varken positief. Op 32% van de bedrijven bleken meer dan 10% van de biggen positief te testen. Bedrijven met zeugen gevaccineerd tegen swine influenza virus (SIV) hadden een significant hoger risico om biggen te hebben die positief waren voor *M. hyopneumoniae* (OR 3,12, 95% CI 1.43 tot 6.83). Het hogere risico in geval van SIV vaccinatie is moeilijk te verklaren, maar het kan te wijten zijn aan het feit dat de varkens op bedrijven met respiratoire symptomen meer kans hebben om te worden gevaccineerd tegen SIV waarbij de mogelijke invloed van andere respiratoire pathogenen zoals *M. hyopneumoniae* over het hoofd gezien wordt. Onze resultaten bevestigen dat infectie met *M. hyopneumoniae* algemeen voorkomt bij 3-weken oude biggen in verschillende Europese landen.

Vaccinatie van biggen is één van de belangrijkste strategieën die momenteel wordt gebruikt voor de bestrijding van *M. hyopneumoniae* infecties. Het is echter niet bekend of vaccinatie van gespeende biggen de verspreiding van *M. hyopneumoniae* tegengaat onder veldomstandigheden tijdens de batterijperiode.

In hoofdstuk 3.2 wordt een studie beschreven naar het effect van vaccinatie tegen *M. hyopneumoniae* op de transmissie bij gespeende varkens onder veldomstandigheden. Tweeënzeventig varkens werden bij het spenen willekeurig toegewezen aan gevaccineerde (V) of niet-gevaccineerde (NV) groepen. Dieren in de V-groep werden gevaccineerd op de leeftijd van 3 weken met een commercieel *M. hyopneumoniae* bacterin vaccin. Bij het spenen en aan het einde van de opfokperiode werd bronchoalveolair spoelvocht genomen. Dat werd onderzocht op de aanwezigheid van *M. hyopneumoniae* door middel van een nested PCR
waarna de transmissieratio van de infectie (Rn) werd berekend. Het percentage positieve varkens in de V en NV groepen bedroeg respectievelijk 14% en 36% bij het spenen en 31% en 64% aan het einde van de opfokperiode. De Rn-waarden voor de V en NV groepen waren respectievelijk 0,71 en 0,56 (P> 0,05). Uit de resultaten van deze studie kan geconcludeerd worden dat vaccinatie de verspreiding van dit respiratoir pathogeen niet significant verminderd.

Vervolgens (hoofdstuk 3.3) werd het infectiepatroon en de ontwikkeling van longlesies bij varkens, geïnfecteerd met een laag of hoog virulente *M. hyopneumoniae* stam, op 4 en 8 weken (w) na infectie (PI) onderzocht. De werkzaamheid van een commercieel geïnactiveerd bacterin vaccin tegen infectie met elk van deze *M. hyopneumoniae* stammen werd ook bepaald. Negentig biggen die vrij waren van *M. hyopneumoniae* werden geselecteerd en 40 van hen werden willekeurig gevaccineerd tijdens hun eerste levensweek. Bij het spenen, werden alle biggen toegewezen aan 10 verschillende groepen en gehuisvest in hokken met HEPA filters. Op de leeftijd van 4 weken, werden de varkens intratracheaal geïnoculeerd met ofwel een hoog virulente of laagvirulente stam van *M. hyopneumoniae*, ofwel met een steriel groeimedium. De helft van alle dieren werd gedood op 4W PI, terwijl de andere helft werd geëuthanaseerd op 8W PI. Dagelijks werd het hoesten geëvalueerd. Na autopsie werden een score van de longlesies, immunofluorescentie (IF), bacteriologische analyse en nested PCR uitgevoerd. Aangetoond werd dat de laag virulente stam, in tegenstelling tot de hoog virulente stam, meer dan 4 weken PI (algemeen aanvaard als het standaard-infectie model) nodig heeft om maximale klinische symptomen te veroorzaken. Vaccinatie verminderde significant de klinische symptomen en de macroscopische en microscopische longlesies bij varkens besmet met de hoog virulente stam. Dit effect was meer uitgesproken na 4 weken PI dan na 8 weken PI. Bij de varkens die besmet waren met de laag virulente stam werd ook een beschermende werking aangetoond, maar het effect was minder uitgesproken dan bij de hoog virulente stam.
Tenslotte werd het effect van een infectie met laag virulente stammen van *M. hyopneumoniae* (LV1 en LV2) op de latere infectie met een hoog virulente stam (HV) geëvalueerd (hoofdstuk 3.4). Vijfenvijftig, 3 weken oude biggen die vrij waren van *M. hyopneumoniae* werden willekeurig toegewezen aan 6 verschillende groepen. Op de leeftijd van 4 weken (D0), werden de groepen LV1-HV-en LV1 intratracheaal geïnoculeerd met LV1 en werden de groepen LV2-HV en LV2 met LV2, en de groep HV met steriel groeimedium geïnoculeerd. Vier weken later (D28) werden de varkens van deze verschillende groepen intratracheaal geïnoculeerd met hetzij de hoog virulente stam (groepen LV1-HV, LV2-HV, HV) hetzij het steriel groeimedium (groepen LV1 en LV2). Een negatieve controlegroep bestond uit varkens geïnoculeerd met steriel groeimedium op D0 en D28. Alle dieren werden 28 dagen na de tweede inenting geëuthanaseerd. Klinische symptomen werden geëvalueerd met behulp van een dagelijkse hoestscore (RDS). Na autopsy werden macroscopische en histopathologische longlesies gekwantificeerd en werden immunofluorescentie (IF) testen op het longweefsel en nested PCR op het BAL-vloeistof uitgevoerd voor de detectie van *M. hyopneumoniae*. Ziektesymptomen en longlesies werden niet waargenomen bij varkens van de negatieve controle groep. In de andere groepen waren er geen of slechts zeer milde klinische symptomen van D0 tot D28. Een significante stijging van de gemiddelde RDS waarden werd echter waargenomen tijdens D28-D56, vooral in de groepen LV1-HV (1,48) en LV2-HV (1,49), in groep HV (0,79), en in mindere mate in de groepen LV1 (0.50) en LV2 (0,65) (P <0,05). De klinische symptomen tussen D28-D56, de longlesies en de intensiteit van de IF kleuring waren meer uitgesproken in de groepen LV1-HV, LV2-HV en HV in vergelijking met de groepen LV1 en LV2. Alle varkens, met uitzondering van die uit de negatieve controlegroep, waren positief met IF testen en PCR op D56. Met deze studie werd aangetoond dat de varkens die ingeënt waren met een laag virulente stam van *M. hyopneumoniae* niet beschermd zijn tegen een volgende infectie met een hoog virulente stam, 4 weken later en
zelfs ernstigere ziekte symptomen ontwikkelen. Dit wijst erop dat opeenvolgende infecties met verschillende *M. hyopneumoniae* stammen kan leiden tot ernstige klinische ziekte op een varkensbedrijf.

Uit het onderzoek beschreven in dit proefschrift, kan worden geconcludeerd dat *M. hyopneumoniae* infecties op jonge leeftijd beginnen en wijdverspreid zijn in de Europese varkensstapel. Vele vragen blijven nog onbeantwoord en nog veel meer gedetailleerde studies zijn nodig om de mogelijke risicofactoren verbonden aan deze infecties te beoordelen.

Er werd geen significant verschil in transmissie van *M. hyopneumoniae* waargenomen tussen biggen die gevaccineerd waren tegen *M. hyopneumoniae* en biggen die niet gevaccineerd waren. Dit betekent dat vaccinatie alleen niet voldoende is om een significante daling van *M. hyopneumoniae* infecties te bekomen en dat aanvullende controlemaatregelen nodig zijn om dit pathogene agens uit de varkensstapel te elimineren. Door middel van toekomstige studies moet nauwkeuriger het aantal *M. hyopneumoniae* bacteriën in de longen gekwantificeerd worden en moet onderzocht worden of bestrijdingsmaatregelen in staat zijn om een significante daling van de infectie in de longen te bekomen.

Het infectiepatroon en verloop zijn verschillend na experimentele infectie met een hoog of laag virulente stam van *M. hyopneumoniae*, en ook de effectiviteit van vaccinatie lijkt te worden bepaald door de aard van de stam. Een beter begrip van het infectiepatroon en de interacties tussen hoog en laag virulente stammen is nodig. Varkens die opeenvolgend geïnfecteerd worden met verschillende stammen ontwikkelen ernstiger klinische symptomen en lesies. Het is echter niet bekend of gelijktijdige infectie met twee verschillende stammen zal leiden tot een ernstiger vorm van de ziekte en of beide stammen zich in hetzelfde tempo in het varken zullen vermenigvuldigen en/of één stam de andere zal domineren. Dit zijn
belangrijke vragen aangezien ze kunnen helpen om het klinisch verloop van de infectie te verklaren en om de huidige controlemaatregelen tegen enzoötische pneumonie te verbeteren.
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CURRICULUM VITAE

Iris Villarreal was born on March 21st 1983, in Warsaw, Poland. Her upbringing had an international background and she spent many years of her childhood in Panama. She finalized her secondary studies in 2001 with an International Baccalaureate diploma from the United World College of the Atlantic in the United Kingdom. Her veterinary studies were undertaken partly at Warsaw University of Life Sciences, Poland, and partly at Università di Bologna, Italy- receiving her DVM diploma in July 2007. During her years of study, she carried out clinical practice on small and exotic animals in countries like Singapore, UK and Panama, as well as a research project on the binding specificity of immunoglobulins and receptors encoded by cytomegalovirus at the University of Cambridge.

Her interest in research led her to start a PhD program, as part of a IWT research project, in January 2008 at the Faculty of Veterinary Medicine in Ghent University. The title of the project was: “Study of the diversity of Mycoplasma hyopneumoniae as basis for a better prevention of enzootic pneumonia in pigs”. The research took place at the Department of Reproduction, Obstetrics and Herd Health and the Department of Pathology, Bacteriology and Poultry diseases.

Iris is first author and co-author of several articles published in international peer reviewed journals. Her experimental work has been presented in different European and international congresses.
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ORAL PRESENTATIONS


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The difference between involvement and commitment is like with

"Ham & Eggs: a day’s work for a chicken, but a lifetime commitment for a pig."