Highly and Low Virulent Mycoplasma hyopneumoniae Isolates: Transmission and Interaction with the Respiratory Tract

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Highly and Low Virulent *Mycoplasma hyopneumoniae* Isolates: 
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‘Success is the ability to go from one failure to another with no loss of enthusiasm’

(Winston Churchill)
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LIST OF ABBREVIATIONS

∞ Infinity
α Recovery Rate
β Infection Rate
ANOVA Analysis of Variance
ATP Adenosine-Tri-Phosphate
BAL BronchoAlveolar Lavage
BALT Bronchus Associated Lymphoid Tissue
CCU Colour Changing Units
DNA DesoxyriboNucleic Acid
DPI Days Post Inoculation
ELISA Enzyme-Linked ImmunoSorbent Assay
EP Enzootic Pneumonia
FS Final Size
GLM Generalised Linear Model
I Infectious
IF Immunofluorescence
IL Interleukin
MALP Macrophage Activating Lipoprotein
MLE Maximal Likelihood Estimator
M. hyopneumoniae Mycoplasma hyopneumoniae
nPCR nested Polymerase Chain Reaction
OD Optical Density
P Infectious Period
PBS Phosphate Buffered Saline
PCR Polymerase Chain Reaction
PCV-2 Porcine Circovirus type 2
PRDC Porcine Respiratory Disease Complex
PRRSV Porcine Respiratory and Reproductive Syndrome Virus
R₀ Basic Reproduction Ratio
Rₐ Adjusted Reproduction Ratio
RDS Respiratory Disease Score
S Susceptible
SEIR Susceptible-Exposed-Infectious-Recovered
SI Susceptible-Infectious
SIR Susceptible-Infective-Removed
SPF Specific Pathogen Free
TLR Toll-Like Receptor
TNF-α Tumor Necrosis Factor-α
CHAPTER 1.

GENERAL INTRODUCTION
1.1. REVIEW OF THE LITERATURE

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the primary pathogen of enzootic pneumonia (EP), a chronic respiratory disease in pigs with a worldwide distribution. *M. hyopneumoniae* is relatively benign in single infections, but in field situations, *M. hyopneumoniae* acts as a door opener for complicated respiratory diseases, and is responsible for important economic losses in modern swine industry (Kobisch and Friis, 1996; Ross, 1999; Maes *et al.*, 2003). The high prevalence of the infection in almost all swine producing areas, together with the significant and long lasting economic losses, contributes to the fact that *M. hyopneumoniae* is one of the most important pathogens in intensive pig production.

As a member of the mollicutes, *M. hyopneumoniae* is a prokaryote organism without cell-wall and it is sensitive to environmental conditions. The organism is characterized by a slow growth *in vitro* and is extremely difficult to isolate (Friis, 1974). Since the first isolations of *M. hyopneumoniae* by Maré and Switzer (1965) and by Goodwin *et al.* (1965), the J-strain, a low virulent strain, was defined as the reference strain of *M. hyopneumoniae*. In the next decades, several investigators were able to prove the existence of differences between field isolates. Ro and Ross (1983) demonstrated heterogenicity by growth inhibition and metabolic inhibition tests among the strains they tested. In 1992, Frey *et al.* showed antigenic differences, while Artiushin and Minion (1996) found different DNA band patterns in Randomly Amplified Polymorphic DNA analysis. Apart from genetic and phenotypic differences, important diversity in virulence among *M. hyopneumoniae* field isolates was only recently described. Inoculation of piglets with different isolates led to major differences in clinical and pathological parameters (Vicca *et al.*, 2003; Strait *et al.*, 2004).

The macroscopic lesions induced by an uncomplicated *M. hyopneumoniae* infection are
characterized by purple to grey, catarrhal pneumonic areas, mainly found bilaterally in the cranial-ventral parts of the lung. They appear from 7 days after experimental infection onwards and reach a maximal size at about 4 weeks after infection (Kobisch et al., 1993). The lesions are dark red to purple and affected lung parts are characterized by a meaty aspect, while a catarrhal exudate is often present in the airways. The bronchial and mediastinal lymph nodes are usually enlarged. The lesions tend to be well demarcated in single infections. Macroscopic lesions are usually resolved 12-14 weeks after infection (Goodwin et al., 1965; Blanchard et al., 1992), but the organism can remain present in the lungs until at least 6 months after infection (Fano et al., 2005a). In the presence of other respiratory pathogens, the lesions become more diffuse and they are sometimes difficult do differentiate from those induced by other pathogens like swine influenza virus or the combination of Pasteurella multocida and Aujeszky’s disease virus (Thacker, 2006).

*M. hyopneumoniae* affects the mucosal clearance system by disrupting the cilia on the epithelial surface and additionally, the organism modulates the immune system of the respiratory tract (Thacker, 2006). This implies that EP is characterized by important interactions with other respiratory bacteria like *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Haemophilus parasuis* and *Streptococcus suis* (Yagihashi et al., 1984; Ciprian et al., 1988; Sørensen et al., 1997). More recently, co-infections and interactions with porcine respiratory and reproductive syndrome virus (PRRSV) (Thacker et al., 1999), Porcine Circovirus type 2 (PCV-2) (Opriessnig et al., 2004) and/or swine influenza (Thacker, 2001a) are frequently described as the ‘Porcine Respiratory Disease Complex’ (PRDC).

Clinical signs and lesions can lead to a suspicion of a *M. hyopneumoniae* infection, but confirmation of the infection can only be made by laboratory testing. Previously, culturing of the organism was described as the gold standard (Friis, 1975). Immunofluorescence (IF)
testing (Kobisch et al., 1978) is also frequently performed. Both tests have limited sensitivity, especially when low numbers of organisms are present. Serology can be used to show presence of the organism on a herd basis, but is not suited for diagnosis on individual animals (Sørensen et al., 1997). At present, (nested) Polymerase Chain Reaction (PCR) testing is seen as the most sensitive tool to detect the organism (Kurth et al., 2002).

Control of the disease is usually based on optimalization of management practices and housing conditions (Maes, 1998). Preventive and curative use of antimicrobials is common, although acquired antimicrobial resistance has been observed (Vicca et al., 2004). Today, vaccination by inactivated bacterin vaccines is worldwide used. These vaccines have been proven to be effective in reducing the clinical symptoms, but only partial protection against the development of lesions is obtained (Haesebrouck et al., 2004).

In this review, literature concerning the transmission and control of M. hyopneumoniae infections and the interaction of the pathogen with the respiratory tract of piglets will be summarized. Additionally, an overview of the literature concerning the experimental quantification of the transmission of infectious diseases will be presented.
1.1.1. TRANSMISSION AND CONTROL OF *Mycoplasma hyopneumoniae*

Since the first description of a chronic pneumonia in pigs by Waldmann and Radtke (1937), EP was initially defined as a ‘virus pneumonia’ (Gulrajani and Beveridge, 1951), because the agent was filterable through a bacterium filter. In 1965, *M. hyopneumoniae* was for the first time isolated from affected lungs by Maré and Switzer (1965) and reproduced after experimental infection by Goodwin *et al.* (1965). During the decades after these first descriptions, mycoplasmal pneumonia has been reported in many countries worldwide (Bäckström and Bremer, 1978; Madec and Kobisch, 1982; Wilson *et al.*, 1986; Pointon *et al.*, 1990; Falk and Liim, 1991; Maes *et al.*, 1999).

At present, *M. hyopneumoniae* has been detected in almost all pig farms in countries with intensive pig production. However, the prevalence largely varies between farms, production systems and countries. The percentage of animals with antibodies at slaughter ranges from 0 to 100% (Maes, 1998). Although some countries were successful in the eradication of *M. hyopneumoniae* at farm or regional level (Switzerland, Denmark, Finland), reinfections of these herds are frequently observed after the sanitation (Jorsal and Thomson, 1988; Hege *et al.*, 2002).
1.1.1.1. Introduction of *Mycoplasma hyopneumoniae* in herds

*M. hyopneumoniae* can be introduced in a herd by different ways. The purchase of subclinically infected pigs (Done, 1996) and airborne transmission between neighbouring herds are regarded as the main sources of introduction (Goodwin, 1985; Stärk *et al.*, 1998). Other transmission routes seem to be less important under field conditions (Whittlestone, 1973).

**Introduction by infected animals**

Introduction of *M. hyopneumoniae* in a farm by import of subclinically infected pigs is by far the most important way of transmission of *M. hyopneumoniae* between farms (Pullar, 1948; Thacker, 2006). In farms free of *M. hyopneumoniae*, there is a risk of introduction of the pathogen, while in farms that are already infected without severe clinical problems, more virulent strains can be introduced and can cause more pronounced symptoms of respiratory disease (Vicca *et al.*, 2003).

Recently, it has been shown that 25% of the Belgian farrow-to-finish and breeding herds have more than once every two months an introduction of breeding animals, while 10% of the farms have approximately once a week an introduction of new animals (Ribbens *et al.*, submitted). Effective prevention of the purchase of subclinically infected animals is hampered by the fact that it is extremely difficult to assure a truly negative status of individual pigs. Serology by enzyme-linked immunosorbent assay (ELISA) is the most common tool to determine the absence of *M. hyopneumoniae* at a herd level (Martelli *et al.*, 2006). However, in herds with a very low infection level, false negative results are easily obtained using only serology. Therefore, it is said that the sensitivity of ELISA is not high enough to declare a
herd free of the infection (Thacker, 2006). This limited sensitivity is due to the fact that \textit{M. hyopneumoniae} is only present on the external surface of the airways, and individually infected animals do not have a uniform serologic response (Erlandson \textit{et al.}, 2005), especially when a low infection pressure is present (Leon \textit{et al.}, 2001). The best method to guarantee freedom of disease on life animals is to assure the absence of \textit{M. hyopneumoniae} DNA in a bronchoalveolar lavage fluid sample by a PCR test (Kurth \textit{et al.}, 2002). However the lavage technique is laborious and requires anaesthesia, which is difficult to perform on a large scale (Hennig-Pauka \textit{et al.}, 2001).

A second important reason for the frequent import of infected animals is the fact that infected animals can remain carrier of the organism without showing clinical disease for at least 6 months after infection (Fano \textit{et al.}, 2005a). Thacker (2001b) even suggested that once infected, animals never completely eliminate the pathogen from the respiratory tract. Nasal swabs from sows, tested by nested PCR, were positive, even in sows of seventh parity (Calsamiglia and Pijoan, 2000). Sørensen \textit{et al.} (1997) could not detect \textit{M. hyopneumoniae} by PCR or by isolation in 200 nasal swabs from experimentally infected animals at 81 days post inoculation, but the organism was cultured from the lungs of these animals.

It can be concluded that replacement stock for specific pathogen free (SPF) herds must be grown up in a herd free of \textit{M. hyopneumoniae} to avoid the risk of introduction of the organism by subclinically infected animals.
Introduction by airborne transmission

Airborne transmission of *M. hyopneumoniae* between farms is regarded as a second important route of disease spreading (Goodwin, 1985; Thomson *et al*., 1992; Morris *et al*., 1995; Stärk *et al*., 1998; Leon *et al*., 2001; Fano *et al*., 2005b). *M. hyopneumoniae* organisms are not resistant in the environment. However, the organism is able to survive and is protected against inactivation in aerosols of respiratory secretions (Goodwin, 1985).

Goodwin (1985) found out that on herds previously free, a higher infection rate was present when an infected herd was located in the neighbourhood. He also found that SPF herds had a fair chance to remain free when they were located at least 3.2 km away from infected herds. Similar results were published by Muirhead *et al*. (1997).

The airborne transmission route is supported by field observations of reintroductions of the organism in SPF herds. In Swiss research, it was found that the distance to infected neighbourhood herds and the transport routes that are regularly used for pig transports, are important risk factors for infection (Stärk *et al*., 1992). The amount of airborne transmission is shown to be relative to the size of the source herd (Thomson *et al*., 1992).

The seasonal pattern that is observed for reinfection of SPF herds, with peak infections between November and March, also supports evidence of airborne transmission (Stärk *et al*., 1992). Winter conditions and higher air humidity can be seen as favourable, since *M. hyopneumoniae* is able to survive in water from 2 to 7°C for more than 30 days (Goodwin, 1985). Dry weather conditions, freezing and rainfall are negatively associated with the occurrence of *M. hyopneumoniae* infections (Stärk, 2000). Wind force and wind direction influence the transmission (Jorsal and Thomson, 1988).
Experimental evaluations of airborne transmission were recently performed by different authors (Stärk et al., 1998; Cardona et al., 2005; Fano et al., 2005b). Stärk et al. (1998) showed presence of *M. hyopneumoniae* by nPCR in filtered air out of infected farms. Cardona et al. (2005) showed the recovery of the bacteria by PCR out of air that was transmitted through a pipeline at 1, 75 and 150 m away from an experimentally produced aerosol containing *M. hyopneumoniae*, but they failed to find viable *M. hyopneumoniae* organisms. Fano et al. (2005b) proved in 2 of the 3 experiments that air out of a barn with experimentally infected animals was able to induce infection in animals placed in a trailer at a distance of 6 m of the barn. Although only a short distance was covered, this was the first experimental proof of airborne transmission of *M. hyopneumoniae* between pigs.

Based upon the experimental and field observations, it can be concluded that airborne transmission of *M. hyopneumoniae* over short distances is an important transmission route. It is the major route of infection for SPF herds where regional eradication has not yet been achieved. Further studies are needed to evaluate the effect of factors such as microbial load, environmental conditions and mixed infections, which can influence this route of transmission.
Introduction by passive vectors

Fomites can possibly act as a mechanical vector of the infection between herds (Goodwin, 1985). Taking into account the fastidious nature of the organism, it can be assumed that standard hygiene protocols are able to prevent transmission between herds. Batista et al. (2002) evaluated the impact of personnel and fomites in the transmission of *M. hyopneumoniae*. They found that hygiene protocols including the use of disposable boots, gloves and coveralls, and a disinfecting footbath at the entrance of the barns were able to prevent transmission to naïve pigs by personnel having weekly contact with infected pigs. Although not demonstrated for *M. hyopneumoniae*, transmission could theoretically also occur by flies and mosquitoes that act in this case as a passive vector of the bacteria. Indeed, research on environmental survival of *Mycoplasma synoviae* showed presence of mycoplasmal DNA in flies and dust by PCR. Additionally, it was indicated that the same isolate was present in 2 consecutive poultry flocks despite of disinfection of the stables during the empty period (Marois et al., 2002). These results indicate that transmission of *M. hyopneumoniae* by passive transport might be possible although this route of transmission is likely to be of minor importance for the introduction of *M. hyopneumoniae* in a herd.

Introduction by semen

This route of transmission should be regarded as very unlikely, since *M. hyopneumoniae* is found to be only present on the cilia of the respiratory airways of infected animals (Mebus and Underdahl, 1977), and has not been reported to multiply in the genital tract of boars. In only one study, performed by Schulman and Estola (1974), *M. hyopneumoniae* was isolated in
one out of 101 semen samples, but Mandrup et al. (1975) were unable to recover *M. hyopneumoniae* out of 169 semen samples. No other studies could confirm the presence of the organism in semen samples.
1.1.1.2. Spread of *Mycoplasma hyopneumoniae* in the farm

Infection occurs mostly by direct contact with infected animals. Newborn piglets can become infected with *M. hyopneumoniae* through contact with lactating sows or gilts which are shedding the organism (Calsamiglia and Pijoan, 2000). This is frequently described as ‘vertical transmission’, although no in-utero or lactogenic transmission occurs (Maes, 1998). A second important route of transmission in the herd is through direct contact with penmates and pigs in close proximity (Clark *et al*., 1993; Morris *et al*., 1995). This route is frequently described as ‘horizontal transmission’. It is not clear which of both is the most important, and considerable variation in infection patterns exists between herds and production systems (Pijoan, 1995; Vicca *et al*., 2002; Sibila *et al*., 2004). Additionally, changes may occur in the transmission patterns of *M. hyopneumoniae* in a herd over time (Fano *et al*., 2006).

Transmission of *Mycoplasma hyopneumoniae* before weaning

Piglets may become infected early in their life time, as already shown by Goodwin *et al*. in 1965 who isolated *M. hyopneumoniae* from piglets before weaning. More recently, this was confirmed by several authors using nPCR on nasal swabs from piglets before weaning. 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 to 13.2% of the piglets positive in a breeding farm at 19 days of age. Sibila *et al*. (2007) performed nasal sampling in piglets from 1 and 3 weeks of age, and the percentage of positive piglets ranged between 0 and 6.4%. Much higher infection rates were obtained by Fano *et al*. (2006). They reported a prevalence between 2.5 and 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 presence of mycoplasmal DNA in the tonsils of one pig and in the bronchial swab of two other pigs.

Infection of the sows by *M. hyopneumoniae* has been demonstrated for the first time by Goodwin et al. (1965). Older sows, especially these in endemic infections, have a lower probability to harbour *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 micro-organism was also demonstrated in nasal samples of older sows (3-7th parity).

Transmission from the sow to the offspring may be influenced by the immune status of the sow. Most probably, in immune sows, shedding of the pathogen will be lower resulting in decreased infection pressure for the piglets. Immune sows will also transmit colostral immunity to their offspring (Rautiainen and Wallgren, 2001). This colostral immunity can reduce the severity of the lesions that are observed, and probably also the infection rates in young animals (Martelli et al., 2006). In a case study of Wallgren et al. (1998), piglets with high titres of maternal antibodies were not likely to show clinical signs in the first two weeks after birth, while piglets in the same herd with low titres of maternal antibodies were showing clinical signs. At present, the protective influence of maternally derived antibodies is not completely elucidated. Protection against severity of disease is attributed to antibodies in sow colostrum (Lam and Switzer, 1971; Wallgren et al., 1998), whereas protection against colonization of the respiratory tract has not yet been proven. This is in agreement with the fact that transmission of *M. hyopneumoniae* cannot be prevented by means of vaccination of the sow (Martelli et al., 2006).
Pig-to-pig transmission during the farrowing period cannot be excluded, but is not likely to be important, since it was found that the onset of shedding by seeder pigs starts only 7 to 14 days post experimental infection, and it could even be delayed in natural infections, where in most cases a lower bacterial load is present (Fano et al., 2005a).

**Transmission of Mycoplasma hyopneumoniae after weaning**

Transmission of *M. hyopneumoniae* throughout the herd is usually slow, and the percentage of *M. hyopneumoniae* seropositive pigs gradually increases towards the end of the fattening period (Maes, 1998). Although pigs can be infected already before weaning, most infections occur after weaning (Vicca et al., 2002; Ruiz et al., 2003). At weaning, 5 to 10% of the animals seems to be infected in most herds (Calsamiglia and Pijoan, 2000; Vicca et al., 2002; Ruiz et al., 2003; Sibila et al., 2007), although considerable variations up to more than 50% are possible (Fano et al., 2006).

Transmission after weaning mainly occurs by direct nose-to-nose contact between infectious and susceptible pigs (Clark et al., 1993). Animals with direct nose-to-nose contact were seven times more likely to become infected, compared to animals with only indirect contact (Morris et al., 1995). Transmission can occur between penmates, independently from their age (Etheridge et al., 1979; Piffer and Ross, 1984). Additionally, indirect contact exposure via air flow between several compartments on the same herd is also important in the transmission of *M. hyopneumoniae*.

Several management and housing factors influence the transmission of *M. hyopneumoniae* in a chronically infected herd.
It has been observed that in continuous production systems, where pigs of different ages are housed together, the highest infection rates are present (Flesja and Solberg, 1981; Clark et al., 1991). This is probably due to the fact that transmission mainly occurs in the growing and finishing units when young and susceptible pigs enter the unit, and share the same airspace with older infected pigs (Gardner and Hird, 1990; Sheldrake et al., 1990).

Open pen-sides are favourable for nose-to-nose contact between different groups of pigs and, therefore, these systems facilitate the transmission of respiratory pathogens between different pens (Morris et al., 1995).

In farms where all-in/all-out production is performed, transmission of *M. hyopneumoniae* between different age groups is delayed. Airborne infections are indeed delayed compared to infections through direct contact (Morris et al., 1995). Additionally, clinical signs and seroconversion are observed later compared with animals that are infected by direct contact exposure, probably due to the lower infection dose (Fano et al., 2005a). In herds with a multi-site production system, as commonly applied in the U.S., the disease also seems to be delayed compared with single-site production herds (Pijoan, 1995).

Peak infection rates are observed in winter when air humidity is high and ventilation is reduced to maintain room temperatures (Stärk, 2000). Other factors like mixing and moving of piglets, high stocking density, low air volume and large herd size contribute to higher infection rates in the farm (Maes, 1998).

Imbalanced herd immunity can also result in a higher transmission of the organism in previously infected herds. Wallgren et al. (1998) described an outbreak of *M. hyopneumoniae* in a previously infected herd, induced by the introduction of two *M. hyopneumoniae*-free boars. It was shown that the newly introduced animals became infected shortly after introduction and were excreting the organism, resulting in a disruption of the herd immunity and a high number of newly infected animals.
The infection dose is also an important factor in the transmission of disease. When high doses were used in experimental infections, clinical signs of coughing started as early as 11 days after infection. With moderate doses, clinical signs only developed after an incubation period of 4 to 6 weeks, while animals infected with low doses developed no or only very mild disease signs (Stevenson, 1998; Fano et al., 2005b). This dose dependent presentation of the disease is also seen in field situations (Kobisch et al., 1993). The onset of shedding after infection seems to be highly variable (Etheridge et al., 1979; Torremorel et al., 2000), which can be responsible for the high variation in infection patterns observed in chronically infected herds (Sibila et al., 2004).

Finally, it has been shown that farms with poor management sometimes show lower infection rates compared to herds with a good quality of management (Vicca et al., 2002). These differences may be due to differences in virulence between *M. hyopneumoniae* isolates (Vicca et al., 2003).
1.1.1.3. Control of *Mycoplasma hyopneumoniae* infections

Factors that influence the clinical impact of *M. hyopneumoniae* in a herd are well described (Maes, 1998; Stärk, 2000) while the factors with an influence on the transmission of the organism are less clear. Although this has not yet been clearly demonstrated, it is reasonable to assume that most of the factors influencing the severity of the disease in a herd also will have an impact on the transmission of *M. hyopneumoniae*.

In the first place, prevention and control of EP are based on improving environmental conditions and management strategies. This includes control of ventilation, temperature and air quality. Also stocking density and air volume availability are important (Maes *et al.*, 1996). Management practices including all in/all out and multi-site production facilitate the control of the disease. The creation of a balanced sow herd with maximal 30% of replacement gilts, minimized contacts with other farms and proper biosecurity measures can prevent the spread and the impact of the infection (Maes, 1998).

Antimicrobials may also be used as an aid in prevention and control of EP. Due to the localisation of the organism on the surface of the respiratory tract, only antimicrobials that are able to reach high concentrations in the mucus and the fluids of the respiratory tract are expected to be effective *in vivo*. It has been demonstrated that chlortetracycline or tylosine in the feed administrated prior to challenge are able to reduce both the severity of the lesions (Vicca *et al.*, 2005) and the infection load (Thacker *et al.*, 2001). Although medication programs proved to be successful in the prevention of economic losses associated with *M. hyopneumoniae* infections (Jouglar *et al.*, 1993; Walter *et al.*, 2000), only partial prevention of the infection was achieved and the organism could not be eliminated from the respiratory tract (Thacker, 2006). Eradication programs where antimicrobials are used in combination with early weaning and the use of multi-site production systems, are able to reduce the number of organisms transmitted from the sows to their piglets (Alexander *et al.*, 1980). One
of the disadvantages of antimicrobial use is that it favours spread of antimicrobial resistance in pathogenic bacteria as well as in bacteria belonging to the microflora of treated animals. Acquired resistance has already been described in *M. hyopneumoniae* field isolates (Inamoto *et al.*, 1994; Vicca *et al.*, 2004).

Inactivated vaccines, produced from adjuvanted whole-cell or membrane preparations are frequently used to control the clinical symptoms and lung lesions induced by *M. hyopneumoniae* infections (Haesebrouck *et al.*, 2004). The exact mechanisms of protection are not yet fully understood, but it has been suggested that mucosal antibodies and cell-mediated immune responses are important (Thacker *et al.*, 2000). The major advantages of *M. hyopneumoniae* vaccination include improvement of the daily weight gain (2-8%) and the feed conversion ratio (2-5%). Additionally, shorter time to reach slaughter weight, reduced clinical signs, lung lesions and lower treatment costs are observed (Maes *et al.*, 1998, 1999).

Although the protection is often incomplete and vaccines do not totally prevent colonization (Thacker *et al.*, 1998; Große Beilage *et al.*, 2005), some studies indicate that the currently used bacterin vaccines may have the ability to reduce the number of organisms in the respiratory tract (Thacker *et al.*, 2004) and may be able to decrease the infection level in a herd. This hypothesis is supported by the fact that maximum beneficial effects of vaccination are reached several months after the initiation of vaccination (Haesebrouck *et al.*, 2004). It has to be evaluated in which way vaccination can reduce transmission of the organism.
1.1.2. Interaction of *Mycoplasma hyopneumoniae* with the porcine respiratory tract

*M. hyopneumoniae* adheres to the ciliated epithelial cells of the porcine respiratory tract which leads to a dysfunction of the mucociliary clearance system (Jacques *et al.*, 1992). In addition, *M. hyopneumoniae* initiates an immune response and modulates the respiratory defence system (Howard and Taylor, 1985). Factors that influence either the adherence or the multiplication of the organism in the respiratory tract, or the interaction of the organism with the immune system, might play a role in the observed difference in virulence between *M. hyopneumoniae* field isolates (Vicca *et al.*, 2003).

1.1.2.1. Colonization of the respiratory tract

*M. hyopneumoniae* colonises the cilia of the trachea, bronchi and bronchioles (Kobisch *et al.*, 1978; Blanchard *et al.*, 1992; Sarradell *et al.*, 2003), without penetration into the lung parenchyma (Doster and Lin, 1988). No other cells of the respiratory tract appear to be colonised (Mebus and Underdahl, 1977). Electron microscopic evaluation of lung tissue from pigs infected with *M. hyopneumoniae* showed that the organism was mainly present at the apex of the cilia and in the intercilliary space, and that it was in close contact with the microvilli (Blanchard *et al.*, 1992; Jacques *et al.*, 1992). Organisms are seldom, and only in low numbers present in non-ciliated bronchioles and alveoli (Baskerville, 1972; Livingston *et al.*, 1972). A study by Kwon and Chae (1999) revealed the presence of *M. hyopneumoniae* DNA mainly in the bronchial and bronchiolar epithelium, although mycoplasmal DNA was also present in alveolar and interstitial macrophages. It is not clear whether *M.*
*hyopneumoniae* really infects these cells, or whether the presence of the DNA was the result of the natural defence system of the macrophages which clean up bacterial organisms, DNA and their products.

Natural or experimental infections via the respiratory tract in conventional pigs have not shown evidence of an extra-pulmonary localisation for *M. hyopneumoniae*. However, the organism was shown to be present in severe chronic arthritis lesions, induced by intravenous injection of the organism (Lloyd and Etheridge, 1981) and was isolated from different internal organs of artificially inoculated specific pathogen free piglets (Marois *et al*., 2007).

The colonisation process has been studied *in vitro* (Jacques *et al*., 1992; Zielinski and Ross, 1992, 1993; DeBey and Ross, 1994; Zhang *et al*., 1994; 1995; Young *et al*., 2000) and *in vivo* (Livingston *et al*., 1972, Mebus and Underdahl, 1977) by several authors. Zielinski and Ross (1993) found out that adhesion of *M. hyopneumoniae* is host specific, and that it is mediated by several proteins and carbohydrates present on the surface of *M. hyopneumoniae* and the cilia. The same authors described the role of non-specific hydrophobic interactions at the start of the attachment process. *M. hyopneumoniae* was shown to specifically bind to the sulphated glycolipid receptor of the respiratory cilia (Zhang *et al*., 1994). Three glycolipids were identified as an adherence receptor (La, Lb and Lc), of which Lc was the strongest binding site (Zhang *et al*., 1994).

Zhang *et al.* (1995) identified the P97 protein to be a ciliary adhesin. The cilium binding activity of this protein was localized to the carboxy terminus of the protein. The P97 gene revealed an operon composed of two open reading frames (R1 and R2) (Hsu and Minion, 1998). The genes encoding the cillum binding site are located in the R1 frame and at least seven AAKPV(E) repeats seem to be required for functional binding (Minion *et al*., 2000). The incomplete inhibition of adhesion by blocking the cilia by P97 antibodies (Chen *et al*., 1998; Hsu and Minion, 1998) supports the hypothesis that other adhesins exist. These may
include a glycoprotein of 110 kDa, consisting of one P54 and two P28 subunits (Chen et al., 1998), a P159 protein that is post-transitionally cleaved in proteins of 27, 51 and 110 kDa (Burnett et al., 2006), as well as a recently described 146 kDa protein (P146) (Stakenborg, 2005). The P146 protein shows homology with both the adhesin P97 and the LppS lipoprotein of *Mycoplasma conjunctivae*. The latter lipoprotein plays a role in attachment of *Mycoplasma conjunctivae* to joint synovial cells in vitro (Belloy et al., 2003).

As the infection progresses, a reduction of ciliary activity, a gradual loss of cilia, microcolony formation and accumulation of *M. hyopneumoniae* at the remaining cells is present. Finally, a complete loss of cilia, destruction and exfoliation of epithelial cells together with an accumulation of viscous exsudate are observed (Blanchard et al., 1992; Jacques et al., 1992; DeBey and Ross, 1994). The loss of mucociliary function is thought to be an important contributor to the increased incidence of secondary bacterial infections associated with *M. hyopneumoniae* infections.

Limited information is available about the specific mechanisms which trigger the ciliary damage. A cytopathic factor was found in the membrane of *M. hyopneumoniae* by Geary and Walczak (1985). This 54 kDa protein induced cytopathogenic effects in human lung fibroblast cell lines. Ciliostasis and loss of cilia quickly ensue through unknown mechanisms (DeBey and Ross, 1994). *M. hyopneumoniae* may increase the intracellular free Ca\(^{++}\) in ciliated epithelial cells, which can induce the loss of cilia (DeBey et al., 1993; Park et al., 2002). Other toxic agents, such as hydrogen peroxide and superoxide radicals produced by *M. hyopneumoniae*, may also contribute to the destruction of the cilia and the damage to epithelial cells.
1.1.2.2. Interaction with the immune system

The complex and chronic appearance of *M. hyopneumoniae* infection and disease seems to be dependent on the evasion and the alteration of the immune response of infected animals (Howard and Taylor, 1985). *M. hyopneumoniae* interacts with the respiratory tract, resulting in both non-specific and specific immune reactions (Razin *et al.*, 1998). Non-specific mechanisms include an increased cytotoxic capacity of macrophages, natural killer cells and T-cells, an enhanced expression of cell receptors, an activation of the complement cascade and a polyclonal activation of B- and T-lymphocytes. Specific protective defence mechanisms consist of stimulation of cell-mediated immunity, opsonisation and phagocytosis of organisms and production of systemic as well as local anti-mycoplasmal antibodies. These immune reactions are important in host defence but have also been shown to play an important role in the development of lesions and the exacerbation of mycoplasmal induced disease. Although many aspects of the immunologic changes induced by *M. hyopneumoniae* infections are still unknown, the immune and inflammatory responses are seen as a major component in respiratory disease (Thacker, 2006).

Cell-mediated immunity was found to be impaired at 15 weeks after *M. hyopneumoniae* infection (Adegboye, 1978). Lymphocyte blastogenesis tests were characterized by a lower incorporation of $^3$H-thimidine in presence of inactivated *M. hyopneumoniae* organisms (Kishima and Ross, 1985). The same authors also showed an immunosuppressive effect of the organism to non-specific mitogens like phytohemagglutinin A. Activity of suppressor T-lymphocytes was increased by *M. hyopneumoniae* (Weng and Lin, 1988). Additionally, decreased phagocytic capacity was observed in pulmonary macrophages that were simultaneously infected with *M. hyopneumoniae* and *A. pleuropneumoniae* (Caruso and Ross, 1990). BAL fluid from pigs experimentally infected with *M. hyopneumoniae* reduced the function of porcine neutrophils, as indicated by a reduced chemiluminescence response (Asai...
Lymphoid hyperplasia around airways and blood vessels was shown when membranes of inactivated *M. hyopneumoniae* were intratracheally given to pigs (Messier, 1986). *M. hyopneumoniae* also provokes a mitogenic response in bronchial and blood lymphocytes (Messier *et al.*, 1990; Messier and Ross, 1991). Additionally, it has been observed that *M. hyopneumoniae* induced pneumatic lesions were less severe in thymectomized pigs treated with antibodies against T-cells (Tajima *et al.*, 1984). In the same study, *M. hyopneumoniae* was reisolated from the spleen of a thymectomized pig. These findings indicate that the cellular response plays a role in development of lesions but is also important in preventing the invasion and the systemic spread of *M. hyopneumoniae*. Alterations to the cellular immune response clearly play an important role in the induction of the disease and the ability of the organism to persist in infected pigs.

In addition to the effects described above, *M. hyopneumoniae* also induces macrophages and monocytes to produce pro-inflammatory cytokines including interleukin (IL)-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and Tumor Necrosis Factor-α (TNF-α) (Asai *et al.*, 1993; 1994; Thacker *et al.*, 2001a; Choi *et al.*, 2006; Muneta *et al.*, 2006; Rodriguez *et al.*, 2007). Inflammatory cytokines mobilize the immune system in response to infections and increase the efficiency of an immune response by enhancing antigen presenting cell function (Murtaugh and Foss, 2002). IL-1 augments the immune response in a number of ways, enhancing the *in vivo* expansion and follicular migration of antigen-stimulated T-cells and providing a unique signal to native CD4⁺ T cells (Murtaugh and Foss, 2002). TNF-α plays an important role as TNF-α levels have been found to be increased rapidly in acute bacterial infections either *in vivo* or *in vitro* (Thanawongnuwech *et al.*, 2001). IL-1 and TNF-α induce a non-specific mitogenic activity on T-lymphocytes and are correlated with the extent of the observed lesions in *M. hyopneumoniae* infected pigs (Rodriguez *et al.*, 2004). IL-2 and IL-4
expressed in numerous mononuclear cells of the bronchus associated lymphoid tissue (BALT) can be incriminated in the lymphoid infiltration, proliferation and maturation resulting, together with other chemical mediators, in the characteristic peribronchiolar lymphoid hyperplasia during EP (Sarradell et al., 2003). IL-6 acts as an anti-inflammatory molecule inhibiting, among others, the expression of inflammatory cytokines, such as TNF-α and IL-1 (Lorenzo et al., 2006). IL-10, produced by T-cells and activated macrophages, is a potent inhibitor of macrophage function and has been associated with clinical protection (Thanawongnuwech et al., 2001). Little is known about the pathophysiolocal role of IL-18, but it seems to be associated with the induction of antibody response (Muneta et al., 2006).

Although the inflammatory response is important in the control of *M. hyopneumoniae* infections, it also plays a role in lesion development. As a matter of fact, the immune reactions and the hyperplasia of the BALT observed after *M. hyopneumoniae* infections seem to be more responsible for the lesions than the organism itself (Thacker, 2006).
1.1.2.3. Virulence factors of *Mycoplasma hyopneumoniae*

Little is known about specific virulence factors of *M. hyopneumoniae*, or about factors involved in the observed differences in virulence between field isolates (Vicca *et al.*, 2003; Strait *et al.*, 2004). The fact that the genome of three different *M. hyopneumoniae* isolates (Minion *et al.*, 2004; Vasconcelos *et al.*, 2005) has recently been sequenced may facilitate the identification of genes and proteins that are important in the induction of disease.

Microscopic investigations of lungs of pigs infected with *M. hyopneumoniae* showed a layer of Ruthenium red staining material enveloping the mycoplasmal cells (Tajima and Yagihashi, 1982). A thinner capsule, induced by *in vitro* passage, was associated with decreased pathogenicity of the organism (Tajima and Yagihashi, 1982). Chen *et al.* (1992) were able to link differences in the composition of the glycoproteins and the glycolipids in the cell membrane of *M. hyopneumoniae* with differences in virulence. These findings suggest that *M. hyopneumoniae* cell envelop components play a role in the pathogenesis of EP, but no information is available about the genomic basis of this specific virulence marker.

Pathogenic strains had a capacity to induce higher intracellular Ca$^{++}$-concentrations in neutrophils compared to non-pathogenic strains (DeBey *et al.*, 1993). These higher Ca$^{++}$-concentrations are also observed in ciliar cells and seem to be responsible for the damage, although the exact mechanism is not known.

*In vitro* isolation of *M. hyopneumoniae* isolates may alter the virulence, depending on the growth medium used and on the number of passages (Zielinski and Ross, 1990). More passages *in vitro* seem to decrease the virulence of the organism (Whittlestone, 1979). DeBey and Ross (1994) showed that *M. hyopneumoniae* strain 232 had a significantly reduced capacity to induce cilliar damage after 20 and 40 passages, compared to the same isolate after only 1 or 2 passages *in vitro*. Hannan *et al.* (1984) showed a clear increase in virulence after 3 *in vivo* passages of the same isolate in gnotobiotic pigs. These findings show that virulence of
M. hyopneumoniae changes relatively quickly. It is not known if these differences were induced by genomic or by phenotypic changes of the organism.

The infection dose also plays a role in the severity of the disease. It has been shown that high doses of inoculum, can induce clinical signs of coughing relatively quickly, while low doses are inducing infection but no or only minimal clinical signs (Stevenson, 1998). It should be elucidated if isolates which differ in virulence have a different capacity to multiply *in vitro* or *in vivo*.

Mycoplasmas are able to avoid clearance by the immune system by changing the cell surface antigens. The number of genes involved in diversifying the antigenic nature of their cell is relatively high compared to other bacteria (Razin *et al.*, 1998). *M. hyopneumoniae* has few significant tandem repeat sequences compared to other mycoplasmas, but one exception is the gene for the cilium adhesin P97. Djordjevic *et al.* (2004) showed differences in proteolytic processing of the P97 cilium adhesin of two different strains. They showed that the non-virulent J strain of *M. hyopneumoniae* is able to selectively cleave its secreted proteins to products of 22, 28, 66 and 94 kDa. The pathogenic strain 232 had in majority similar products, although the 22 and 28 kDa protein were further processed in this strain.
1.1.3. **MODELLING THE TRANSMISSION OF INFECTIOUS DISEASES**

The most important parameter in the study of disease transmission and notably in comparing population dynamical effects of control strategies is the Basic Reproduction Ratio, $R_0$ (Heesterbeek, 2002). In veterinary epidemiology, $R_0$ is defined as the expected number of secondary infections arising from one typically infectious animal during its entire infectious period (de Jong and Diekmann, 1992). It is undoubtedly “one of the most valuable ideas that mathematical thinking has brought to epidemic theory” (Heesterbeek and Dietz, 1996). Nowadays, the concept is frequently used in analytic and experimental veterinary epidemiology to study the transmission dynamics of infectious diseases and to quantify the effect of several interventions on the transmission (de Jong and Kimman, 1994; Bouma *et al.*, 1995; 1996; Dewulf *et al.*, 2001, Velthuis *et al.*, 2003). $R_0$-values have already been calculated for a number of viral and protozootic agents, and for some bacterial diseases, both in human and veterinary medicine (Dietz, 1993), but not yet for *M. hyopneumoniae*. 
1.1.3.1. Definition

As a general definition, \( R_0 \) is the expected number of secondary individuals produced by an individual during its lifetime, although the definition of ‘secondary’ strongly depends on the context. In ecology and social demography, \( R_0 \) is seen as the lifetime reproduction success of an individual (Heffernan et al., 2005). In veterinary epidemiology however, the basic reproduction ratio, \( R_0 \), is defined as the expected number of secondary infections arising from a single typical individual during his or her entire infectious period, in an infinite population of susceptible individuals (de Jong and Diekmann, 1992). Thus \( R_0 \) is a measure of the potential spread of an infectious agent in a susceptible population.

\( R_0 \) is influenced by the duration of the infectious period, the probability of infecting a susceptible individual during a contact and the average number of susceptible individuals contacted in each time period (Dietz, 1993). The concept can be used to evaluate the transmission of infections between individuals in a herd, e.g. classical swine fever inside a farm (Dewulf et al., 2001), or to evaluate the transmission of diseases between herds in a region e.g. Avian Influenza (Stegeman et al., 2004) and Foot-and-Mouth disease (Ferguson et al., 2001). In the latter case, the herd is the unit, and the R-value (commonly described as \( R_{HI} \)) gives the number of secondary farms that became infected due to an infectious farm.

From the definition, it follows that in populations where \( R_0 < 1 \), each infected individual produces, on average, less than one new infected individual. By consequence, it predicts that the infection will not be able to survive in the population and after a certain period, no new infections will occur and the disease will fade out. This is called ‘the threshold theorem’ (Diekmann et al., 1990).

When \( R_0 > 1 \), the pathogen can spread in the population. The relationship between the \( R_0 \)-value and the expected fraction of infected animals in a population is presented in Fig. 1 (de Jong, 1995). From this figure, it is clear that a dramatic change in the final fraction of infected
animals is observed. If $R_0 < 1$, the final infected fraction will be 0; while if $R_0 = 4$, almost 100% of the population will became infected. When an infectious agent with an $R_0 > 1$ enters a susceptible population, the most likely fraction of infected animals at the end of the outbreak is demonstrated in Fig. 1. Nevertheless, there is also a certain probability that the infection will fade out in the early stage (minor outbreak). The probability of such a minor outbreak is inversely related to the $R_0$ ($P_{\text{minor outbreak}} = 1/R_0$) (de Jong, 1995).

Figure 1. The threshold theorem: the final size, fraction of eventually infected animals in a population plotted against the reproduction ratio $R_0$ (de Jong, 1995).
1.1.3.2. History

The concept of the basic reproduction ratio was originally developed for demographic studies (Böckh 1886; Sharp and Lotka, 1911; Dublin and Lotka, 1925; Kuczynski, 1928) and was used to investigate vector-borne diseases, such as malaria (Ross, 1911; MacDonald, 1952) and for directly transmittable human and animal infections (Kermack and McKendrick, 1927; Dietz, 1975; Hethcote, 1975).

At present, numerous studies have been performed to study and to quantify the transmission of infectious diseases. Often, these studies are based on observational data from epidemic or endemic diseases. The magnitude of the $R_0$-value is used to estimate the risk on an epidemic or a pandemic of emerging diseases. For instance, $R_0$ of Severe Acute Respiratory Syndrome (SARS) was estimated to understand the potential danger of this disease (Lipsitch et al., 2003). $R_0$ has also been estimated to characterize the spread of Bovine Spongiform Encephalitis (BSE) (Ferguson et al., 1999); Food-and-Mouth disease (Ferguson et al., 2001) and novel strains of Avian Influenza viruses (Stegeman et al., 2004). The spread of Malaria (Hagmann et al., 2003), Ebola (Chowell et al., 2004) and Scrapie (Gravenor et al., 2004) have also been described by means of $R_0$.

Quantitative estimation of the transmission ratio based on experimental data has recently gained interest in veterinary epidemiology. Although the first experiments to quantify the transmission of infectious diseases in mice were already performed by Greenwood et al., in 1936, the first paper in which the $R_0$ was estimated based on experimental data and the effect of an intervention on the transmission was quantified, was only published in 1994 by de Jong and Kimman. In the following years, several experiments were performed to quantify transmission of mainly viral agents (Table 1). Only very recently, transmission of bacteria such as *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) (Velthuis et al., 2003) and F4+ *Escherichia coli* (*E. coli*) (Geenen et al., 2004) were quantified by transmission
experiments. In most cases, in a first experiment the disease spread is characterized and the infection model is fitted to the specific situations, while in consequent experiments, different approaches are used to compare the spread of different strains or to evaluate the impact of control measures like vaccination.

Table 1. A chronological overview of the estimations of transmission parameters under experimental conditions for animal pathogens (for each infectious agent only the first publication is given).

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Animal</th>
<th>Transmission Parameter</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aujeszky’s disease virus</td>
<td>Swine</td>
<td>$R_0 = 10.0$</td>
<td>de Jong and Kimman, 1994</td>
</tr>
<tr>
<td>Infectious bronchitis virus</td>
<td>Poultry</td>
<td>$R_0 = 19.95$</td>
<td>de Wit et al., 1998</td>
</tr>
<tr>
<td>Classical swine fever virus</td>
<td>Swine</td>
<td>$R_0 = 81.3$</td>
<td>Laevens et al., 1998</td>
</tr>
<tr>
<td>Bovine herpes virus vaccine</td>
<td>Cattle</td>
<td>$R_0 = 0$</td>
<td>Mars et al., 2000</td>
</tr>
<tr>
<td>Sarcopte scabiei var. suis</td>
<td>Swine</td>
<td>$\beta^a = 0.056$</td>
<td>Stegeman et al., 2000</td>
</tr>
<tr>
<td>PRRSV</td>
<td>Swine</td>
<td>$2 &lt; R_0 &lt; \infty$</td>
<td>Nodelijk et al., 2001</td>
</tr>
<tr>
<td>Encephalomyocarditis virus</td>
<td>Swine</td>
<td>$R_0 = 1.24$</td>
<td>Maurice et al., 2002</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>Swine</td>
<td>$0.033 &lt; \beta^b &lt; \infty$</td>
<td>Velthuis et al., 2003</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>Poultry</td>
<td>$1.3 &lt; R_0 &lt; \infty$</td>
<td>van der Goot et al., 2003</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>Poultry</td>
<td>$0.4 &lt; R_0 &lt; \infty$</td>
<td>Heres et al., 2004</td>
</tr>
<tr>
<td>F4+ <em>E. coli</em></td>
<td>Swine</td>
<td>$R_0 = 7.1$</td>
<td>Geenen et al., 2004</td>
</tr>
<tr>
<td><em>Campylobacter</em> spp.</td>
<td>Poultry</td>
<td>$\beta^a = 1.04$</td>
<td>van Gerwe et al., 2005</td>
</tr>
<tr>
<td><em>Mycoplasma gallisepticum</em></td>
<td>Poultry</td>
<td>$4.5 &lt; R_0 &lt; \infty$</td>
<td>Feberwee et al., 2005</td>
</tr>
<tr>
<td>Foot-and-mouth disease virus</td>
<td>Cattle</td>
<td>$R_0 = 2.52$</td>
<td>Orsel et al., 2005</td>
</tr>
</tbody>
</table>

$^a$ $\beta^a$ = average number of new infestations per infested animal per day

$^b$ $\beta = $ average number of infections by one infectious animal
1.1.3.3. Modelling disease transmission

In quantitative epidemiology, mathematical models are used to describe the dynamics of disease transmission. The choice of an appropriate mathematical model is important since it must mimic the true biology as accurate as possible. In any case, it must be considered that a mathematical model is always simplifying the true transmission pattern (Kroese and de Jong, 2001). A commonly used stochastic model to describe disease transmission is the ‘Susceptible-Infective-Removed or Recovered’ (SIR)-model (Bailey, 1975). This model assumes that a susceptible animal can become infected. Thereafter, the animal becomes infectious for a more or less defined period. Once the animal has recovered, it becomes fully immune and cannot become infected or infectious again. Additionally, it is assumed that the probability that an animal infects a susceptible animal is constant during its infectious period. Depending on the pathogen under consideration, this model can be adapted to e.g. ‘Susceptible-Infectious’ (SI)-models in cases where no recovery or immune status is reached, or a ‘Susceptible-Exposed-Infectious-Recovered’ (SEIR)-model when animals are not infectious for a certain period after exposure to the agent. It can be derived from the pathogenesis of several infectious agents that other models are possible for diseases where animals become infected more than once by the same pathogen (without the induction of protective immunity) or where animals are going through a latent phase without being infectious during that period (Becker, 1979).

In the SIR model, the number of contact infections in a population is proportional to the number of Infectious animals \(I\), the probability of contact between infectious and Susceptible \(S\) animals, the total number of animals in the population \(N\), the duration of the infectious period \(P\) and the chance of transmission when a contact occurs (infection rate \(\beta\)) (de Jong et al., 1995). The infection rate in a given population is \(\beta SI/N\). The recovery rate \(\alpha\)
can be estimated as $1/P$. The $R_0$-value can be calculated as $R_0 = \beta/\alpha$ (Velthuis et al., 2002). In some situations, the infection rate and the recovery rate can be estimated separately and they can both be used in the evaluation of the effect of control measures (de Jong et al., 1996). For example, when vaccination is applied, it can result in a lower excretion of the organism by infectious animals ($P$ is decreased), in an increased resistance of susceptible animals ($\beta$ is decreased) or most likely in a combination of both. Prevention of excretion ($P = 0$) will theoretically result in $\alpha = \infty$ and consequently in $R_0 = 0$. On the other hand, when a vaccinated animal cannot be infected after exposure, $\beta = 0$ and consequently $R_0 = 0$. Both $\alpha$ and $\beta$ are valuable tools to estimate the effects of interventions during the transmission of a disease. In comparison to traditional vaccination-challenge experiments, where the effect of the reduced susceptibility is usually demonstrated, transmission experiments provide the unique possibility to quantify the combined effect of a reduction on infectivity and on susceptibility of the vaccinated animals.

Until recently, most of the transmission experiments were performed to investigate the transmission of viral agents. For these infections, the SIR-model is well suited to describe the epidemic pattern and it is easy to obtain data about the infection moments of individual animals. For example, observations in transmission experiments of classical swine fever clearly demonstrate that, after infection, animals become infectious very fast, and after a relative short period, the animals are removed out of the experiment (Dewulf et al., 2001). The definition of the infectious period is assessed by the measurement of the viraemic period. However, in transmission experiments with bacterial diseases, like *A. pleuropneumoniae* (Velthuis et al., 2003), the duration of the infectious period is highly variable and difficult to determine. Additionally, animals can carry the bacteria in their tonsils long before they become infectious (Velthuis, 2002).
1.1.3.4. Estimating transmission parameters

When the appropriate mathematical model is selected, one needs to have data to estimate the transmission parameters ($\alpha$, $\beta$ and $R_0$). Data to estimate the transmission of an infectious agent can be obtained from field observations, where the information about infection and recovery of animals is obtained by sampling animals during an outbreak or by performing transmission experiments. In these experiments, a number of susceptible animals ($S$) are placed together with a number of infectious animals ($I$). During the experiment, data are generated about the infection and/or recovery of all $S$ and $I$ animals. In general, the experiment ends when $I = 0$ or $S = 0$ (Kroese and de Jong, 2001). In Table 2 a comparison of the advantages of both approaches is presented.

Table 2. Major differences between experimental transmission studies and transmission data obtained from the field.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Experimental conditions</th>
<th>Field data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection route</td>
<td>Experimentally</td>
<td>Natural</td>
</tr>
<tr>
<td></td>
<td>Controlled</td>
<td>Not controlled</td>
</tr>
<tr>
<td>Infectious dose</td>
<td>Mostly higher than natural</td>
<td>Natural</td>
</tr>
<tr>
<td>Infectivity</td>
<td>Constant</td>
<td>Variable</td>
</tr>
<tr>
<td>Timing of infection</td>
<td>Controlled</td>
<td>Unknown</td>
</tr>
<tr>
<td>Diagnostic procedures</td>
<td>Defined</td>
<td>Limited</td>
</tr>
<tr>
<td>Timing of diagnostics</td>
<td>Defined</td>
<td>Variable</td>
</tr>
<tr>
<td>Reliability of data</td>
<td>All factors are controlled</td>
<td>Unknown</td>
</tr>
<tr>
<td>Number of animals</td>
<td>Limited</td>
<td>High</td>
</tr>
<tr>
<td>Interactions with other diseases</td>
<td>Not present</td>
<td>Variable and unknown</td>
</tr>
<tr>
<td>Generalization of the conclusions</td>
<td>Difficult</td>
<td>Depends on field situations</td>
</tr>
</tbody>
</table>
Until today, three different methods have been described to calculate the infection parameters based upon experimental data: Generalised Linear Models (GLM) (Becker 1989; de Jong et al., 1996); Martingale Estimations (de Jong and Kimman, 1994) and Maximal Likelihood Estimation based on the final size (FS) (Kroese and de Jong, 2001). The selection of one of the three approaches depends on the data that are available. The advantage of the FS method is that only the number of infected animals at the end of the process is needed. No exact data are needed for the timing of infection and the moment of recovery. Based on the FS, Kroese and de Jong (2001) described the use of the Maximal Likelihood Estimator (MLE) to calculate the $R_0$ with the highest probability to fit with the observed outcomes of the experiments. The FS approach may be used to estimate the transmission of bacterial diseases like *M. hyopneumoniae* during a defined period, since no recovery is assumed shortly after infection (Fano et al., 2005a).

Accurate diagnostic procedures are important since they are used to classify animals as ‘infected’, ‘infectious’ or ‘recovered’. Depending on the method (GLM or Martingale estimator) used to estimate $R_0$, an intensive sampling procedure may be needed to determine the number of new infections and the duration of the infectious period. When an FS method is used, the classification of the animals must be accurate at the end of the experiment, while information during the experiment is not essential.

In transmission experiments, the number of animals is often limited due to practical, financial and ethical reasons (Kroese and de Jong, 2001) while data from field observations are in general more extended. It has been shown that a situation with $S = I$ provides the most accurate estimation when $R_0$ is around 1 (de Jong, 1995). The number of animals used in one experimental unit does not influence the point estimate of $R_0$ since the models are taking into account $I$ and $S$ at the start of the experiment (Diekmann et al., 1990). Additionally, the magnitude of the $R_0$ is independent of the size of the populations in which it was estimated.
(Bouma et al., 1995), although the use of several replicates is very useful to avoid false conclusions based on extreme situations which can occur due to chance.

To be able to generalize conclusions from the experimental data to field situations, one needs to create circumstances in the experimental setting which are as comparable as possible to the field situation. Preferably, inoculation has to result in a certain infectious state, which is comparable to the infection state in naturally infected animals (Kroese and de Jong, 2001). This is the case in field situations, but experimental inoculation usually results in increased infectivity (Velthuis et al., 2002). In transmission experiments, the timing of infection is controlled, while in field observations, this depends on the natural infection pattern. During experimental transmission studies, the experimental circumstances are under control and can be standardized. Only the investigated factor is different between the two compared populations. By consequence, more insight can be obtained about causative factors which contribute to the transmission. However, transmission estimates based on field observations have the advantage that conclusions can be generalized more easily. Finally, in experimental settings, it is possible to investigate transmission of diseases which are not present in the field, or to test interventions with unlicensed products before they are applied on a large scale with the unknown effects on the population.

In conclusion, it can be stated that the best way to study the effect of possible interventions is to test them first in controlled experiments, which are as close as possible to reality. Consequently, when the factor has experimentally been proven to reduce transmission in a satisfying way, the intervention can be evaluated in a field setting. It can be determined which control measures, and at what magnitude, are most effective in reducing $R_0$ below 1. The successful strategy of the use of transmission experiments and the possibility to use the conclusions in the field is very properly demonstrated when strategies in reducing $R_0$ of Aujeszky’s disease virus below 1 (de Jong and Kimman, 1994) were evaluated.
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Review of the Literature


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associated lymphoid tissue of pigs naturally infected with *Mycoplasma hyopneumoniae*. Vet Pathol. 40, 395-404.


1.2. AIMS OF THE STUDY

*Mycoplasma hyopneumoniae* is the primary pathogen of enzootic pneumonia, a disease that is worldwide present in the swine industry. Recently, isolates of *M. hyopneumoniae* with highly divergent virulence have been described. Although knowledge about the epidemiology and the transmission of *M. hyopneumoniae* infections has been extensively published, when we started our study, the transmission of *M. hyopneumoniae* had not yet been evaluated and quantified in an experimental setting. Possible differences in the transmission of highly and low virulent isolates were not yet evaluated. Additionally, no information was available about differences in the interaction of different *M. hyopneumoniae* isolates with the porcine respiratory tract.

The general aims of the present thesis were to obtain better insights in the transmission of *M. hyopneumoniae* and in the interaction of this micro-organism with its porcine host.

The specific aims of the present thesis were:

1. To evaluate and to compare the transmission of highly and low virulent *M. hyopneumoniae* isolates in nursery piglets.

2. To evaluate the effect of vaccination with a conventional bacterin vaccine on the transmission of a highly virulent *M. hyopneumoniae* isolate in nursery piglets.

3. To characterize possible differences in the interaction of highly and low virulent *M. hyopneumoniae* isolates with the respiratory tract of gnotobiotic piglets.
CHAPTER 2.

EXPERIMENTAL STUDIES
CHAPTER 2.1.

**POPULATION DYNAMICS OF *MYCOPLASMA HYOPNEUMONIAE***
Experimental Studies

CHAPTER 2.1.1.

QUANTIFICATION OF THE SPREAD OF *Mycoplasma hyopneumoniae* IN NURSERY PIGS USING TRANSMISSION EXPERIMENTS

Tom Meyns, Dominiek Maes, Jeroen Dewulf, Jo Vicca,
Freddy Haesebrouck, Aart de Kruijf

ABSTRACT

*M. hyopneumoniae* is present in almost all swine herds worldwide, but transmission of the pathogen through herds is not yet fully clarified. The aim of this study, performed in 2003, was to investigate and to quantify the transmission of *M. hyopneumoniae* under experimental conditions by means of an adjusted reproduction ratio (R\textsubscript{n}). This R\textsubscript{n}-value, calculated according to the final size method, expresses the mean number of secondary infections due to one typical infectious piglet during the nursery period. The period lasted from 4 to 10 weeks of age, corresponding with the nursery period used in most European production systems. Additionally, a comparison was made between transmissions of highly virulent and low virulent isolates.

Forty-eight weaned piglets, free of *M. hyopneumoniae*, were housed in 6 separate pens. During 6 weeks, 2 animals experimentally infected with *M. hyopneumoniae* were housed together with 6 susceptible piglets. At the end of the study, the number of contact-infected animals was determined by the use of nPCR on bronchoalveolar lavage fluid. The R\textsubscript{n}-values of the highly virulent and the low virulent isolates were estimated to be 1.47 (0.68 - 5.38) and 0.85 (0.33 - 3.39), respectively. No significant difference between the groups was found (*P* = 0.53). The overall R\textsubscript{n} was estimated to be 1.16 (0.94 - 4.08). Under the present experimental conditions, the transmission of *M. hyopneumoniae*, assessed for the first time by a reproduction ratio, shows that one piglet infected before weaning will infect on average one penmate during the nursery phase.
1. INTRODUCTION

*M. hyopneumoniae* is considered the primary pathogen associated with enzootic pneumonia (EP) (Goodwin *et al.*, 1965; Ross, 1999) or porcine respiratory disease complex (Ross, 1999; Thacker *et al.*, 1999). Both diseases cause important economic losses to the swine industry due to reduced growth rate, poor feed conversion ratio and increased susceptibility to infections with other organisms (Ross, 1999; Maes *et al.*, 2003). At the present time, control is mostly accomplished by the use of management strategies, medication and/or vaccination (Mateusen *et al.*, 2001), but optimal timing of the control measures remains a critical point. Knowledge of precise timing of the infection and complete clarification of the transmission of *M. hyopneumoniae* throughout the herd would make it possible to control the disease in a more efficient way.

It is known that immunologically naïve piglets between 3 and 12 weeks old are equally susceptible to infections with *M. hyopneumoniae* (Piffer and Ross, 1984), and in many herds piglets become infected during the nursery period (Vicca *et al.*, 2002; Ruiz *et al.*, 2003). It has been shown that weaned piglets can infect each other during the nursery period (Clarck *et al.*, 1991), but quantification of the infection rate has not yet been performed.

The spread of several viral agents like classical swine fever (Laevens *et al.*, 1998; Dewulf *et al.*, 2001), pseudorabies virus (de Jong and Kimman, 1994; Bouma *et al.*, 1996) and porcine reproductive and respiratory syndrome virus (PRRSV) (Nodelijk *et al.*, 2001) has already been quantified by means of a reproduction ratio. However, transmission experiments for the quantification of the spread of bacterial diseases have not been frequently used until today. Recently, Velthuis *et al.* (2003) successfully designed an experimental setting to describe the transmission of *Actinobacillus pleuropneumoniae*. 
The purpose of this study was to investigate and quantify the transmission of *M. hyopneumoniae* under experimental conditions among weaned piglets, by calculating an adjusted reproduction ratio (R<sub>a</sub>). Since previous work has clearly shown differences in virulence between *M. hyopneumoniae* isolates (Vicca et al., 2003), we also investigated possible differences in transmission between groups infected with a highly virulent and a low virulent *M. hyopneumoniae* isolate, respectively.

2. MATERIALS AND METHODS

2.1. Experimental design

Forty-eight, 3 week-old, cross-bred (Rattlerow Seghers®, Buggenhout, Belgium), castrated male 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 source herd was a high health breeding herd in which repeated serological monitoring of sows and pigs of different age categories had been performed during the last 5 years. During that time, there was no clinical or serological evidence of *M. hyopneumoniae* infection, and no lung lesions typical for *M. hyopneumoniae* infection were observed in slaughter pigs. The experiment was performed in 2003. Animals were randomly assigned into 2 groups at arrival. The animals were fed a commercial feed without antimicrobials and were allowed to acclimatize for 1 week. At 28 days of age, within each group, 6 pigs (seeder pigs) were randomly selected for inoculation with a highly virulent or a low virulent *M. hyopneumoniae* isolate. Two infected animals were placed in each of the 6 isolated pens: 3 pens for the pigs inoculated with a highly virulent isolate and 3 pens for the
pigs inoculated with a low virulent isolate. The inoculation day was designated as day 0 post-infection (0 DPI). At 2 DPI, 6 susceptible contact-animals were added to each pen. Each group of 8 piglets was housed in a pen equipped with absolute filters (HEPA U15) to avoid spread of *M. hyopneumoniae* and other pathogens between the groups. The disease spread was monitored during a period of 6 weeks, corresponding with the duration of the nursery period mostly applied under field conditions in Western Europe. The study was performed after approval of the Ethical Committee for animal experiments of the Faculty of Veterinary Medicine, Ghent University.

2.2. *M. hyopneumoniae* inocula and intratracheal inoculation

Two Belgian *M. hyopneumoniae* field isolates, obtained from the lungs of pigs from 2 different Belgian farrow-to-finish herds, were used. These isolates had been proven to be highly virulent and low virulent during earlier comparative virulence studies (Vicca *et al.*, 2003). The highly virulent isolate (F7.2C) was obtained from a herd experiencing clinical symptoms associated with EP and could be filter cloned after 10 passages *in vitro*. The low virulent isolate (F13.7B) was obtained from an infected herd without clinical symptoms associated with EP and could be filter cloned after 9 passages *in vitro*.

Twelve pigs were anaesthetized intramuscularly with 0.22 ml/kg of a mixture of Xylazin (Xyl-M 2%®, VMD, Arendonk, Belgium) and Zolazepam and Tiletamin (Zoletil® 100, Virbac, Louvain la Neuve, Belgium), and inoculated intratracheally with 7 ml inoculum, containing \(1 \times 10^7\) CCU/ml of the highly virulent or the low virulent *M. hyopneumoniae* isolate (Vicca *et al.*, 2003). Success of inoculation was assessed by the results of seroconversion and by a nested polymerase chain reaction (nPCR) results on bronchoalveolar lavage (BAL) fluid.
2.3. Clinical observations

Each group of pigs was observed daily for 15 min. Body condition, appetite, presence of dyspnea and tachypnea were recorded. In addition, a clinical respiratory disease score (RDS) was assessed daily (Halbur et al., 1996). RDS could range from 0 to 6: 0 (no coughing), 1 (mild coughing after an encouraged move), 2 (mild coughing while leaving the pigs undisturbed), 3 (moderate coughing after encouraged move), 4 (moderate coughing while leaving the pigs undisturbed), 5 (severe coughing after encouraged move), 6 (severe coughing while leaving the pigs undisturbed). Summation of coughing scores was made and a median respiratory score, with inter-quartile range, was calculated for the 42 days of the trial.

2.4. Serology

Blood samples from all pigs were taken upon arrival, at 23, 32 and 43 DPI to detect antibodies against *M. hyopneumoniae*, using the DAKO® Mh ELISA (DAKO, Glostrup, 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. The blood samples taken upon arrival and at 43 DPI were also checked for PRRSV antibodies in the serum by means of the HerdChek® PRRS ELISA (Idexx Laboratories, Westbrook, ME, USA).
2.5. Lung lesions, immunofluorescence, histopathology and bacteriological examination

Macroscopic and typical *M. hyopneumoniae* induced pneumonic lesions were quantified using a lung lesion score diagram (Hannan *et al.*, 1982). Total lung scores could vary between score 0 (no lesions) and a theoretical maximum of 35. A sample of the left lung was used to perform a semi-quantitative immunofluorescence assay (score 0 - 3) to detect the presence of *M. hyopneumoniae* (Kobisch *et al.*, 1978). In addition, tissue samples of each left lung were taken for histopathological examination. Samples were collected on the edge of lung lesions, if present. In case no macroscopic lesions were present, the sample was collected from the left cranial lung lobe. The percentage of lung area occupied by air (percentage of air) was examined by means of an image analysis system (Optimas® 6.5, Media Cybernetics, Silver Spring, USA). Using light microscopy, the severity of peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation consistent with *M. hyopneumoniae* induced pneumonia lesions, were scored (Morris *et al.*, 1995). A sample of each left lung was inoculated on Columbia agar supplemented with 5% sheep blood (Oxoid, Hampshire, UK) with a *Staphylococcus intermedius* streak for support of *Actinobacillus sp.* growth, and on Columbia CNA agar (Oxoid, Hampshire, UK). Plates were incubated overnight in a 5% CO₂-enriched environment at 37°C, and identification of isolated bacteria was performed as described by Quinn *et al.* (1994).

2.6. BAL and nPCR

All piglets were euthanized at 43 DPI by deep anaesthesia with 0.3 ml/kg of a mixture of Xylazin (Xyl-M 2%, VMD, Arendonk, Belgium) and Zolazepam and Tiletamin (Zoletil® 100, Virbac, Louvain la Neuve, Belgium), followed by exsanguination. The lungs were
removed and the BAL was performed on the right lung to detect the presence of *M. hyopneumoniae* organisms with a nPCR-test (Stärk *et al.*, 1998). Every right lung was washed with 50 ml phosphate buffered saline (PBS), and the recovered fluid was centrifuged at 4000g during 30 min. The pellet of the lavage fluid was resuspended in 1 ml PBS and stored at -80°C until DNA extraction was performed. DNA was extracted using a DNA easy Kit (Westburg, The Netherlands), and a nPCR test was performed on the extract. If BAL fluid was found positive by nPCR, the animal was considered to be infected.

2.7. Statistical analysis

Estimation of the transmission of *M. hyopneumoniae* in each group was done using a stochastic infection model. Therefore, we assumed that the process of transmission of *M. hyopneumoniae* among the piglets was in accordance with the Susceptible-Infectious (S-I) model. We assumed that once an animal was infected, it did not recover before the end of the trial and remained infectious. Given that the population exists of (S,I) animals, after an infection occurred, it consists of (S-1,I+1) animals. In the model, the number of contact infections, determined by the number of positive BAL fluids at the end of the trial, was the observed variable (*X*_i). *X*_i is also called the ‘final size’ of the outbreak. Because the final size is a discrete stochastic variable, it can only attain whole numbers, and each of these has its own probability. Using the algorithm described by de Jong and Kimman (1994), we calculated the probability distribution of the final size for the given parameters and start conditions. This distribution only depended on the *R*_0-value (Diekmann *et al.*, 1990), and is defined as the mean number of secondary cases caused by one typical infectious pig during the nursery period. In our study, we had 6 populations with *N* = 8 animals, where initially 2 animals were infectious (*I*_0 = 2) and 6 animals were susceptible (*S*_0 = 6) (non-infected
contact-exposed pigs). In the experiment, the probability distribution of the final size was represented by \( F(X_i \mid R_n, N, S_0, I_0) \).

We used the maximum likelihood estimator (MLE) to assess the \( R_n \)-values of the highly virulent and low virulent groups. This MLE is calculated numerically from:

\[
R_{n,\text{MLE}} = \max_{R_n} \prod_{i=1}^{m} F(X_i, R_n \mid N, S_0, I_0)
\]

in which \( F(X_i, R_n \mid N, S_0, I_0) \) is the likelihood function for the observed value \( X_i \), when \( N, S_0 \) and \( I_0 \) are given and \( m \) is the number of experiments.

A linear mixed effect model (S-plus), using pen as random variable, was used for the analysis of clinical symptoms and histopathological results. Lung lesion scores were compared using non-parametric analysis of variance (SPSS 11.0). Time until first seroconversion was analysed by Cox regression survival analysis (SPSS 11.0). Results of inoculated pigs and contact pigs were always analysed separately. 1 Statistical results were considered to be significant when P-values were lower than 0.05. Data were analysed using the statistical packages SPSS 11.0 (SPSS 11, SPSS Inc. Illinois, USA) and S-Plus 6.1 (Insightful Corp., Seattle, USA).
3. **RESULTS**

3.1. Major parameters

a. nPCR on BAL

All animals experimentally infected with *M. hyopneumoniae* were positive for *M. hyopneumoniae* in the BAL by nPCR-testing at 43 DPI. In the highly virulent group, 10 out of 18 (56%) contact piglets were found positive for *M. hyopneumoniae*, while in the low virulent group, 6 out of 18 (33%) contact animals were found positive for *M. hyopneumoniae*. The distribution in each pen of contact piglets that became contact-infected is shown in Table 1.

Table 1: Distribution of infection\(^a\) in 6 different nursery pens measured by nPCR in BAL\(^b\) fluid in a Belgian study of transmission of *M. hyopneumoniae*, 2003.

<table>
<thead>
<tr>
<th># of infected animals / # of susceptible animals</th>
<th>Highly virulent <em>M. hyopneumoniae</em></th>
<th>Low virulent <em>M. hyopneumoniae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pen 1</td>
<td>2/6</td>
<td>Pen 4</td>
</tr>
<tr>
<td>Pen 2</td>
<td>3/6</td>
<td>Pen 5</td>
</tr>
<tr>
<td>Pen 3</td>
<td>5/6</td>
<td>Pen 6</td>
</tr>
<tr>
<td>Pen 4</td>
<td>2/6</td>
<td></td>
</tr>
<tr>
<td>Pen 5</td>
<td>2/6</td>
<td></td>
</tr>
<tr>
<td>Pen 6</td>
<td>2/6</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Infection was measured 43 days post infection

\(^b\) bronchoalveolar lavage
b. Reproduction Ratio

The $R_0$-value (95% confidence interval) was 1.47 (0.68 - 5.38) and 0.85 (0.33 - 3.39) for animals infected with the highly virulent and the low virulent isolate, respectively. Since no significant difference was found between the $R_0$-value in these two groups ($P = 0.53$), the overall $R_0$-value was calculated, by combining the outcomes of all the six transmission experiments. This overall $R_0$-value was 1.16 (0.94 - 4.08).

3.2. Minor parameters

a. Clinical symptoms

Pigs had their first positive coughing score on average at 14.96 DPI, with a spread of 4 - 31 DPI. All piglets had at least one day with a positive coughing score. First positive coughing score was noted on average at 14.56 DPI (5 - 28 DPI) and at 18.89 DPI (11 - 31 DPI), for contact pigs in the highly virulent and the low virulent group, respectively. Piglets inoculated with the highly virulent isolate had a first positive coughing score at 6.83 DPI (4 - 10 DPI), while animals inoculated with the low virulent isolate had the first coughing score later, at 10.83 DPI (7 - 13 DPI).

The median (Interquartile Range) respiratory score for the inoculated animals was 85.0 (66.5 - 136.5) and 34.0 (19.5 - 40.5) in the highly virulent and in the low virulent groups, respectively ($P < 0.05$). The median respiratory score for the contact pigs was 16.0 (9.8 - 23.3) and 8.0 (6.0 - 12.3) in the highly virulent and in the low virulent groups, respectively ($P > 0.05$).
One of the susceptible piglets in the highly virulent group had symptoms of meningitis. This piglet was treated with amoxycillin and non-steroidal anti-inflammatory drugs, and recovered after treatment. No other piglets showed clinical symptoms apart from coughing.

b. Serology

Upon arrival, all piglets were serologically negative for \textit{M. hyopneumoniae} and PRRSV. The results of the serological examination for \textit{M. hyopneumoniae} at 23, 32 and 43 DPI are presented in Table 2. At the end of the study, none of the piglets had antibodies against PRRSV. Although highly virulent isolates induced an earlier seroconversion, with lower OD-values (data not shown) compared with the low virulent isolates, time until first seroconversion was not significantly different between both inoculated groups ($P = 0.147$).

Table 2: Number of nursery pigs (%) serologically\textsuperscript{a} positive for \textit{M. hyopneumoniae} at different days post infection in a Belgian transmission study, 2003.

<table>
<thead>
<tr>
<th></th>
<th>Days post infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23</td>
</tr>
<tr>
<td>Highly virulent isolate</td>
<td></td>
</tr>
<tr>
<td>Inoculated (n=6)</td>
<td>4 (67%)</td>
</tr>
<tr>
<td>Contact pigs (n=18)</td>
<td>0</td>
</tr>
<tr>
<td>Low virulent isolate</td>
<td></td>
</tr>
<tr>
<td>Inoculated (n=6)</td>
<td>0</td>
</tr>
<tr>
<td>Contact pigs (n=18)</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} assessed by use of DAKO\textsuperscript{®} Mh ELISA at 23, 32 and 43 days post infection.
c. Lung lesion scores

The mean lung lesion scores of the inoculated pigs were 8.50 and 1.93 for animals experimentally infected with a highly virulent and a low virulent isolate, respectively ($P > 0.05$). The mean lung lesion scores of the contact animals were 0.61 and 0.18 in the highly virulent and low virulent groups, respectively ($P > 0.05$). These scored lung lesions, red to dark red consolidations, were typically for a *M. hyopneumoniae* infection. Five out of the 18 contact piglets in the highly virulent group and 4 of 18 contact piglets in the low virulent group had macroscopic lung lesions. Besides lesions indicative for *M. hyopneumoniae* infection, one contact infected piglet had pleuritis in the low virulent group, and 5 out of 24 animals had pleuritis and/or pericarditis in the highly virulent group.

d. Bacteriology

In the highly virulent group, *Streptococcus suis* (*S. suis*) was isolated from the lungs of 19 of the 24 animals and *Actinobacillus minor* (*A. minor*) from the lungs of one animal. In the low virulent group, *S. suis* was isolated from the lungs of 16 of the 24 animals, *A. minor* from the lungs of 2 animals and *Pasteurella multocida* from the lungs of one animal.

e. Immunofluorescence and histology

*M. hyopneumoniae* was demonstrated by immunofluorescence testing in the lungs of 5 of the 6 pigs intratracheally inoculated with the highly virulent isolate, and in one of the 6 pigs intratracheally inoculated with the low virulent isolate. Only one out of the 18 contact pigs in each group was found positive. The average score of infiltration of inflammatory cells around
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respiratory airways and blood vessels was 1.00 and 0.73 for the inoculated pigs in the highly virulent and low virulent groups, respectively ($P > 0.05$). The percentages of air in the lungs were 55.70 and 52.63 for inoculated pigs in the highly virulent and the low virulent groups, respectively ($P > 0.05$).
4. DISCUSSION

The purpose of this study was to measure the spread of *M. hyopneumoniae* in nursery pigs and to calculate an adjusted reproduction ratio (Rₐ) based on the outcome of the transmission experiments. It is believed that once infection is established in a few pigs, transmission among penmates occurs, especially after animals are pooled together at weaning time (Ross, 1999). According to what was expected based on the results of previous experiments (Etheridge et al., 1979) and based on data from field observations (Vicca et al., 2002), we were able to demonstrate transmission in all experimental groups during the nursery period. Rₐ-values can certainly be used to evaluate differences in spread between groups with a different treatment (e.g. vaccination or medication) or to evaluate the influence of certain control strategies on the spread of *M. hyopneumoniae*.

One of the main problems in transmission experiments for bacterial diseases is to determine the exact duration of the infectious period, because diagnostic tools are limited to define the onset and the end of an infectious period for most bacteriological infections and especially for *M. hyopneumoniae*. Therefore we calculated an Adjusted Reproduction Ratio. This Rₐ-value differed from the conventional Basic Reproduction Ratio (R₀) because we did not take into account the entire infectious period, but only a strictly limited observation period. This was done because it was not possible to determine the onset and the end of the infectious period of animals infected with *M. hyopneumoniae* in our trial. In this experiment we only used the infectious time during the nursery period and therefore we could not be sure that the entire infectious period was included. In consequence, the calculated reproduction ratio is only valid for this specified period. The nursery period is particularly important because a few animals, which became infected before weaning, could infect a considerable number of other piglets during this period. In that way, several piglets become infected with
*M. hyopneumoniae* before the finishing period, which might result in long-lasting and considerable economic losses attributed to mycoplasmal pneumonia (Maes *et al.*, 2003). In our model, an $R_n$-value of 1 means that one animal, infected before the nursery period, will result on average in one contact infected animal during the nursery period. The overall $R_n$-value of 1.16 indicates that the spread of *M. hyopneumoniae* is not excessive, but in general the infection will be maintained and will even result in more infectious animals at the end of the nursery period. Based on positive findings of *M. hyopneumoniae* in sows up to the seventh parity (Calsamiglia and Pijoan, 2000), it is reasonable to expect that fattening pigs infected with *M. hyopneumoniae* will remain infected during the rest of the fattening period, and might infect other animals.

The difference in transmission between the highly virulent and the low virulent isolate was not statistically significant, but there was a tendency towards a more intensive spread in the highly virulent group. We used 48 piglets in the present experiment, but to find a significant difference, more pigs should be used in this experiment. The difference in spread is possibly due to a higher load of mycoplasmas in the respiratory tract of these animals. Although this hypothesis was not investigated, the faster seroconversion after inoculation and the more intense immunofluorescence, also point to a more effective multiplication in the lungs of the highly virulent *M. hyopneumoniae* strain. The results concerning clinical, macroscopical and serological investigations were necessary to confirm that we really do deal with two strains of different virulence. These results of the present study confirmed results of previous experiments (Vicca *et al.*, 2003) concerning the difference of the highly virulent and the low virulent strains, but the number of inoculated animals was too limited to find significant results for all parameters.

We used the positive results in the BAL fluid for the calculation of the $R_n$ because this is believed to be the most sensitive parameter to predict an infection with *M. hyopneumoniae* at
individual level (Kurth et al., 2002). Nasal swabs were not used since results are less reliable. The nasal cavity is not the natural location for *M. hyopneumoniae*, so it is possible to obtain negative nasal swabs, even if infectious *M. hyopneumoniae* is present in the lungs. On the other hand, it is also possible to obtain positive nasal swabs, because only mycoplasmal DNA and not viable or infectious *M. hyopneumoniae* organisms are detected with PCR. Serological diagnosis relies upon the response of animals to the organism, and after experimental infection, pigs show positive antibody response with DAKO® Mh ELISA after 2-8 weeks (Sørensen et al., 1997). This highly variable and extended period is not accurate enough to predict the infection status in this experiment. This is also clear in our results where only 75% of the inoculated pigs had seroconverted by the end of the trial.

The design of our study differs in several ways from the situation occurring in commercial pig herds. Firstly, the experimental infection occurred as late as two days before the start of the experimental period. The inoculated animals became fully infectious after one or two weeks (Sørensen et al., 1997). In situations where piglets become infected during the first days of their life, they will be fully infectious during the whole nursery period and this will possibly result in a higher transmission. Also the volume of air available per pig is mostly smaller in commercial herds compared with the situation in this experimental design, which could possibly have led to a lower transmission in our experiment. On the other hand, the seeder pigs were inoculated with a relatively high dose of *M. hyopneumoniae* organisms. This high dose might not be reached under natural conditions and could have increased the transmission. The fact that only eight animals were used in one experimental unit did presumably not influence the $R_n$-values since the transmission was calculated taking into account the initial number of infectious and susceptible animals in the experiment (Diekmann et al., 1990). In a transmission experiment with pseudorabies virus, Bouma et al. (1995) showed that there was no difference in the estimated transmission ratio based upon
experiments with different numbers of infectious and susceptible animals at the start of the study.

5. **CONCLUSION**

This is the first time that reproduction ratios are calculated for *M. hyopneumoniae*. Based upon the findings in this experiment, we can conclude that, under the present experimental conditions, pigs infected with *M. hyopneumoniae* will infect on average one penmate during the nursery period.

**ACKNOWLEDGEMENTS**

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CHAPTER 2.1.2.

COMPARISON OF TRANSMISSION OF *Mycoplasma hyopneumoniae* IN VACCINATED AND NON-VACCINATED POPULATIONS

Tom Meyns, Jeroen Dewulf, Aart de Kruijff, Dries Calus, Freddy Haesebrouck, Dominiek Maes

ABSTRACT

A transmission experiment was performed to quantify the effect of vaccination on the transmission of *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) in nursery piglets by means of an adjusted reproduction ratio (*R<sub>n</sub*>). Thirty piglets, vaccinated at one week of age, and 30 non-vaccinated piglets, free of *M. hyopneumoniae*, were housed in 6 separate pens. In each pen, three animals that were intratracheally inoculated with *M. hyopneumoniae*, were housed together with 7 contact piglets during the conventional nursery period of 6 weeks. At the end of the study, the infectious status of the animals was determined based on results of nPCR performed on bronchoalveolar lavage fluid. The *R<sub>n</sub>*-value in the vaccinated group was 2.38 (1.07 - 7.53) while in the non-vaccinated group, an *R<sub>n</sub>*-value of 3.51 (1.51 - 9.34) was observed, both not significantly different from each other (*P* = 0.77). Under the actual experimental conditions, transmission of *M. hyopneumoniae* in nursery piglets was only numerically lower in vaccinated groups. In addition, vaccination with a conventional vaccine could not prevent the establishment of *M. hyopneumoniae* organisms in the lung.
1. **INTRODUCTION**

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the primary pathogen of enzootic pneumonia (Goodwin *et al*., 1965). The disease causes important economic losses to the swine industry due to reduced performance of the pigs (growth rate, feed conversion ratio) and to increased use of antimicrobials (Ross, 1999; Maes *et al*., 2003). At the present time, control is mostly accomplished by improvement in management practices, the use of medication and vaccination. The currently used vaccines consist of adjuvanted whole-cell preparations and have extensively been proven to be effective in reducing the clinical symptoms and the lung lesions associated with *M. hyopneumoniae* infections (Maes *et al*., 1999; Jensen *et al*., 2002), but only a partial protection against development of lung lesions is obtained (Keich *et al*., 2001). Although the protection is often incomplete and vaccines do not totally prevent colonisation (Thacker *et al*., 1998; Grosse Beilage *et al*., 2005), some studies indicate that commercial vaccines may have the ability to reduce the number of *M. hyopneumoniae* organisms in the respiratory tract (Thacker *et al*., 2004) and to decrease the infection level in a herd. The latter hypothesis is supported by the fact that the maximum beneficial effect of vaccination is reached several months after initiation of vaccination (Haesebrouck *et al*., 2004).

Modern pig producers strive to increase the productivity of their herds and are constantly looking to maintain or to achieve an optimal health status of their animals. In this respect, there is an increasing interest of the pig industry for elimination of pathogens such as *M. hyopneumoniae* from infected herds or production systems. Quantitative knowledge on the transmission capacity of *M. hyopneumoniae* and the effects of vaccination on this transmission is a prerequisite if eradication of the pathogen is aimed.
Transmission experiments, in which the spread of a pathogen is compared in a standardized way between vaccinated and non-vaccinated populations, are a very efficient tool to investigate possible beneficial effects. Such experiments have successfully been used to evaluate vaccination-induced reduction in transmission of viral swine pathogens like Pseudorabies virus (de Jong and Kimman, 1994; Bouma et al., 1997) and classical swine fever virus (Dewulf et al., 2001). To date, transmission experiments for the quantification of the spread of bacterial pathogens in pigs are rare. Recently, the transmission of a low and a highly virulent isolate of *M. hyopneumoniae* in non-vaccinated nursery piglets was evaluated (Meyns et al., 2004). In the present study, the same experimental model was used to investigate and to quantify the possible influence of vaccination on the transmission of a highly virulent *M. hyopneumoniae* isolate in nursery piglets.
2. MATERIALS AND METHODS

2.1. Experimental design

Sixty, one week-old, cross-bred (Rattlerow Seghers®, Buggenhout, Belgium), male and female piglets from 8 different sows were obtained from a herd that was free of *M. hyopneumoniae* and PRRSV. The source herd was a high health breeding farm in which repeated serological monitoring of sows and pigs of different age categories was performed during the last 6 years. During that time, there was no clinical or serological evidence of a *M. hyopneumoniae* infection, and no lung lesions typical for *M. hyopneumoniae* infection were observed in the slaughter pigs.

The animals were randomly divided into two groups of 30 piglets. Piglets of group 1 were vaccinated in the neck with 2ml of an inactivated *M. hyopneumoniae* whole-cell vaccine (Stellamune One®, Pfizer Animal Health S.A., Louvain-la-Neuve, Belgium). The piglets of the other groups were injected with 2ml of sterile saline. All piglets were weaned at 21 days of age, and moved to the animal facilities of the Faculty of Veterinary Medicine, Ghent University, Belgium. The animals were fed a commercial feed without antimicrobials and were allowed to acclimatize during 5 days. At 26 days of age, 18 seeder pigs (9 vaccinated and 9 non-vaccinated) were randomly selected for inoculation with a highly virulent *M. hyopneumoniae* isolate. They were housed in 6 separated pens: 3 pens for the vaccinated pigs and 3 pens for the non-vaccinated pigs with 3 animals in each pen. This inoculation day was designated as day 0 post-infection (0 DPI). At 2 DPI, 7 vaccinated or non-vaccinated contact-animals were added to each pen. Each group of 10 piglets was housed in a pen equipped with absolute filters (HEPA U15) to avoid spread of *M. hyopneumoniae* or other pathogens between the groups. Possible transmission of *M. hyopneumoniae* was monitored during a
period of 6 weeks, corresponding with the duration of the nursery period mostly applied under field conditions in Western Europe. The study was performed after approval of the Ethical Committee for animal experiments of the Faculty of Veterinary Medicine, Ghent University.

2.2. *M. hyopneumoniae* inoculation

A highly virulent Belgian *M. hyopneumoniae* field isolate was used. The isolate (MhF7) was obtained from a herd experiencing clinical symptoms associated with EP and could be filter cloned after 10 passages *in vitro* (Vicca *et al.*, 2002).

Eighteen pigs were anaesthetized intramuscularly with 0.22 ml/kg of a mixture of Xylazin (Xyl-M 2%, VMD, Arendonk, Belgium) and Zolazepam and Tiletamin (Zoletil® 100, Virbac, Louvain la Neuve, Belgium), and inoculated intratracheally with 7 ml inoculum, containing $1 \times 10^7$ CCU/ml of the *M. hyopneumoniae* isolate. Success of inoculation was assessed by the results of seroconversion and by the results of a nested polymerase chain reaction (nPCR) test on bronchoalveolar lavage (BAL) fluid.

2.3. Clinical observation

The same person observed each group of pigs daily for 15 min. Body condition, appetite, presence of dyspnea and tachypnea were recorded. In addition, a clinical respiratory disease score (RDS) was assessed daily (Halbur *et al.*, 1996). RDS could range from 0 to 6: 0 (no coughing), 1 (mild coughing after an encouraged move), 2 (mild coughing while leaving the pigs undisturbed), 3 (moderate coughing after encouraged move), 4 (moderate coughing while leaving the pigs undisturbed), 5 (severe coughing after encouraged move), 6 (severe coughing while leaving the pigs undisturbed). A mean respiratory score was calculated for the 42 days of the trial. General health and daily weight gain were measured during the study.
2.4. Serology

Blood samples from all pigs were taken at vaccination, and at 7, 23, 29, 36 and 44 DPI to detect antibodies against *M. hyopneumoniae*, using the DAKO® Mh ELISA (DAKO, Glostrup, 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. Sera from 44 DPI were also checked for PRRSV antibodies in the serum by means of the HerdCheck® PRRS Elisa (Idexx Laboratories, Westbrook, ME, USA).

2.5. Lung lesions, immunofluorescence (IF)-testing and bacteriological examination

Macroscopic pneumonic lesions were quantified using a lung lesion score diagram (Hannan et al., 1982). Total lung scores could vary between score 0 (no lesions) and a theoretical maximum of 35. A sample of each lobe of the left lung was used to perform a semi-quantitative IF assay (score 0 - 3) to detect the presence of *M. hyopneumoniae* (Kobisch et al., 1978). Samples were collected at the edge of lung lesions, if present. A sample of each BAL fluid 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). Plates were incubated overnight in a 5% CO₂-enriched environment at 37°C and identification of isolated bacteria was performed as described by Quinn et al. (1994).
2.6. Bronchoalveolar lavage, nasal swabs and nPCR

All piglets were euthanized at 44 DPI by deep anaesthesia with 0.3 ml/kg of a mixture of Xylazin (Xyl-M 2%®, VMD, Arendonk, Belgium) and Zolazepam and Tiletamin (Zoletil® 100, Virbac, Louvain la Neuve, Belgium), followed by exsanguination. The lungs were removed and the bronchoalveolar lavage (BAL) was performed on the right lung half to detect the presence of *M. hyopneumoniae* organisms with a nPCR-test (Stärk *et al.*, 1998). Every right lung half was washed with 50ml phosphate buffered saline (PBS), and the recovered fluid was centrifuged at 4000g during 30 min. The pellet of the lavage fluid was resuspended in 1ml PBS and stored at -80°C until DNA extraction was performed. DNA was extracted using a DNA easy Kit (Westburg, The Netherlands), and a nPCR test was performed on the extract. If BAL fluid was found positive by nPCR, the animal was considered to be infected.

Nasal swabs were collected every week from all animals, and from intratracheally inoculated animals twice a week during the first 3 weeks after experimental inoculation. DNA was extracted with the DNA easy kit and nPCR was performed on the extract.

2.7. Statistical analysis

$R_0$-values were used to compare the difference in transmission between the 2 groups. Estimation of the transmission of *M. hyopneumoniae* in each group was done using a stochastic infection model. Therefore, we assumed that the process of transmission of *M. hyopneumoniae* among the piglets was in accordance with the Susceptible-Infectious (S-I) model. We assumed that once an animal was infected, it did not recover before the end of the trial and remained infectious. Given that the population exists of (S,I) animals, after an infection occurred, it consists of (S-1,I+1) animals. In the model, the number of contact
infections, determined by the number of positive BAL fluids at the end of the trial, was the observed variable ($X_i$). $X_i$ is also called the ‘final size’ of the outbreak. Because the final size is a discrete stochastic variable, it can only attain whole numbers, and each of these has its own probability. Using the algorithm described by de Jong and Kimman (1994), we calculated the probability distribution of the final size for the given parameters and start conditions. This distribution only depended on the $R_n$-value, and is defined as the mean number of secondary cases caused by one typical infectious pig during the nursery period. We used the maximum likelihood estimator to assess the $R_n$-values (Meyns et al., 2004) of the vaccinated and non-vaccinated groups.

A linear mixed effect model, using pen as random variable, was used for the analysis of clinical symptoms. Lung lesion scores and IF-scores were compared using non-parametric analysis of variance. Statistical results were considered to be significant when P-values were lower than 0.05. Data were analysed using the statistical packages SPSS 12.0 (SPSS 12, SPSS Inc. Illinois, USA) and S-Plus 6.1 (Insightful Corp., Seattle, USA).
3. Results

3.1. Major parameters

a. nPCR test on BAL

All animals intratracheally inoculated with *M. hyopneumoniae* were positive for *M. hyopneumoniae* in the BAL by nPCR-testing at 44 DPI. In the vaccinated group, 16 out of 21 (76%) contact piglets were found positive for *M. hyopneumoniae*, while in the non-vaccinated group, 19 out of 21 (90%) contact animals were found positive for *M. hyopneumoniae* (Table 1).

Table 1: Distribution and number of contact infected pigs in the different pens measured by nPCR in BAL fluid 44 DPI and Rn-values in both vaccinated and non-vaccinated groups.

<table>
<thead>
<tr>
<th></th>
<th>Vaccinated Pigs</th>
<th>Non-vaccinated Pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pen 1</td>
<td>6/7*</td>
<td>2.38 (1.07-7.53)**</td>
</tr>
<tr>
<td>Pen 2</td>
<td>3/7</td>
<td>3.51 (1.51-9.34)**</td>
</tr>
<tr>
<td>Pen 3</td>
<td>7/7</td>
<td></td>
</tr>
<tr>
<td>Pen 4</td>
<td>7/7</td>
<td></td>
</tr>
<tr>
<td>Pen 5</td>
<td>5/7</td>
<td></td>
</tr>
<tr>
<td>Pen 6</td>
<td>7/7</td>
<td></td>
</tr>
</tbody>
</table>

*number of infected animals / number of susceptible animals

**Rn-value with 95% Confidence interval
b. Adjusted Reproduction Ratio

The $R_n$ values (95% confidence interval), based on results of nPCR positive BAL fluids, were 2.38 (1.07 - 7.53) and 3.51 (1.51 - 9.34) for the vaccinated and non-vaccinated groups, respectively. No statistically significant difference was found between the $R_n$-value in these two groups ($P = 0.77$).

3.2. Minor parameters

a. Clinical symptoms and general health

Intratracheally inoculated pigs in the vaccinated and non-vaccinated groups started to cough on average (spread) at 11.22 DPI (7 - 20 DPI) and 10.78 DPI (7 - 18 DPI), respectively. In the intratracheally inoculated pigs, coughing was observed for at least 6 days in the vaccinated group, and for at least 8 days in the non-vaccinated groups.

The mean respiratory score (95% confidence interval) for the inoculated animals was 0.57 (0.39 - 0.75) and 1.29 (0.82 - 1.76) in the vaccinated and non-vaccinated groups, respectively ($P < 0.05$). Results of contact piglets are presented in Table 2.

Daily weight gain (95% CI) between 21 and 70 days of age was 406g (371 - 441) for all piglets in the non-vaccinated group, and 429g (395 - 464) for all piglets in the vaccinated group ($P > 0.05$).

No piglets died during the study and clinical symptoms different from coughing were not observed.
b. nPCR on nasal samples

On average, 2.44 of the 9 investigated nasal swabs were positive in the vaccinated and intratracheally inoculated animals, while the average number of positive nasal swabs in the non-vaccinated animals was 3.00.

Table 2: Summary of diagnostic results in vaccinated and non-vaccinated contact piglets of a *M. hyopneumoniae* transmission study.

<table>
<thead>
<tr>
<th></th>
<th>Vaccinated animals (n=21)</th>
<th>Non-vaccinated animals (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coughing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animals with at least one pos. observation</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>Average number of pos. days</td>
<td>2.24</td>
<td>4.86</td>
</tr>
<tr>
<td>Mean score</td>
<td>0.22</td>
<td>0.52</td>
</tr>
<tr>
<td>Macroscopic lung lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of pos. animals</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Mean score</td>
<td>0.18*</td>
<td>1.95*</td>
</tr>
<tr>
<td>IF score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of pos. animals</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>Mean score</td>
<td>0.20*</td>
<td>0.58*</td>
</tr>
<tr>
<td>Nasal swabs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean number of pos. swabs</td>
<td>0.62</td>
<td>1.29</td>
</tr>
</tbody>
</table>

* Results are significantly different ($P < 0.05$)

c. Serology

Upon arrival at the animal facilities of the Faculty, all non-vaccinated piglets were serologically negative for *M. hyopneumoniae*, while 9/30 of the vaccinated animals had already detectable antibodies. At the end of the experiment (44 DPI), all vaccinated animals except 2, remained seropositive. One of these seronegative animals had a negative BAL fluid, no macroscopic lesions and no positive nasal swabs.

In the non-vaccinated group, all intratracheally inoculated animals had antibodies against *M. hyopneumoniae* at 23 DPI. Two, 6 and 9 out of the 21 contact piglets had seroconverted at 29, 36 and 44 DPI, respectively.

In none of the sera, antibodies against PRRSV were detected.
d. Lung lesion scores

The mean lung lesion score of the inoculated pigs were 0.30 and 4.41 for intratracheally inoculated animals of the vaccinated and the non-vaccinated groups, respectively ($P < 0.05$).

The average lung lesion score of the vaccinated contact piglets were 0.18 while the non-vaccinated contact piglets had an average lung lesion score of 1.95 ($P < 0.05$) (Table 2).

e. Immunofluorescence

*M. hyopneumoniae* was demonstrated by IF-testing in the lungs of 8 of the 9 inoculated pigs in the vaccinated group with an average score of 0.81, and in all inoculated pigs in the non-vaccinated group with an average score of 1.33 ($P = 0.08$).

The average IF-scores of the vaccinated contact piglets were 0.20 while the non-vaccinated contact piglets had an average a score of 0.58 ($P < 0.05$) (Table 2).

f. Bacteriology

In the vaccinated group, *Haemophilus parasuis* (*H. parasuis*) was isolated from BAL fluid of 8 animals, *H. parasuis* and *Pasteurella multocida* (*P. multocida*) were isolated from BAL fluid of one animal, and from BAL fluid of another animal *H. parasuis*, *P. multocida* and *Streptococcus suis* (*S. suis*) were isolated. In the non-vaccinated group, bacteria could be isolated from BAL fluids of 23 animals: *H. parasuis* (n=14), *P. multocida* (n=6), *H. parasuis* and *S. suis* (n=1) and *H. parasuis*, *P. multocida* and *S. suis* (n=2).
4. DISCUSSION

The transmission ratios obtained in the present study, namely 2.38 and 3.51 in vaccinated and non-vaccinated nursery pig populations, respectively, imply that a vaccinated piglet infects on average 2.38 penmates during the nursery phase, while a non-vaccinated piglet will infect on average 3.51 penmates. A comparable but slightly lower $R_n$-value (1.47 (0.68 - 5.38)) was obtained with the same highly virulent isolate in a previous transmission experiment in non-vaccinated piglets (Meyns et al., 2004). The difference between both experiments may be due to a faster onset of infection, since in the present experiment, the first seropositive contact infected pigs were already present at 29 DPI, whereas in the previous experiment, seropositive contact infected pigs were detected for the first time at 43 DPI. These results once again confirm that, due to biological variation, subsequent transmission experiments performed under highly comparable conditions seldom provide exactly the same point-estimates of $R_n$-values. Therefore, interpretation of the confidence intervals is more informative.

It has been shown that *M. hyopneumoniae* vaccination could significantly improve the performance of grow-finishers pigs and reduces the clinical symptoms and lung lesions (Maes *et al.*, 1999; Jensen *et al.*, 2002). Maes *et al.* (2003) demonstrated that vaccination is economically justified, even in herds with a low infection pressure. In several field studies, however, the number of vaccinated seropositive pigs gradually increased towards the end of the finishing period, indicating indirectly that *M. hyopneumoniae* can still circulate in vaccinated pigs (Le Grand *et al.*, 1996; Maes *et al.*, 1999). The results of the present study confirm all these field observations since it was observed in the vaccinated population that both the efficacy parameters were clearly improved and that simultaneously $R_n$ was significantly larger then 1. The later finding indicates that vaccination of pigs with the current
vaccines will not lead to the eradication of the infection on a herd. Therefore, if eradication is attempted, vaccination will be a tool but additional control measures will be necessary.

When comparing the $R_n$ in the unvaccinated (3.51) and vaccinated groups (2.38), a numerical but no significant reduction in transmission is observed. Transmission does not only depend on the infectivity of infectious animals, but also on the susceptibility of not yet infected animals (de Jong and Kimman, 1994). These kinds of experiments, where both the seeder and contact animals were vaccinated, are the most appropriate to measure the full effect of vaccination on the transmission. It also mimics the field situation where, under normal circumstances, all animals in a group are vaccinated. As a result it is difficult to determine whether the slightly lower $R_n$-values in the vaccinated group results from a reduced shedding of the seeder pigs or an increased resistance against colonization in the contact pigs. Most likely it is a combination of both. The less intense IF scores and the lower number of nPCR positive nasal samples in the inoculated vaccinated animals in comparison to the inoculated unvaccinated animals, suggests that the number of *M. hyopneumoniae* organisms in the lungs of the vaccinated animals was lower compared to the unvaccinated animals, which probably results in a lower excretion. Similar but less pronounced differences were found in the contact animals, suggesting a reduced susceptibility in the vaccinated piglets. Nevertheless, the relative small and statistically not significant reduction in the transmission of *M. hyopneumoniae* leads us to the conclusion that vaccination had only a limited effect on transmission under the actual experimental conditions.

The source herd of the animals used in the experiment was free of *M. hyopneumoniae* and PRRSV, but we found a surprisingly high number of animals infected with *H. parasuis*, *P. multocida* and *S. suis* at the end of the experiment. Since fewer vaccinated animals were infected (8/30) with *H. parasuis* compared to the number of non-vaccinated animals (17/30), it is possible that pigs suffering from clinical *M. hyopneumoniae* infections are more
susceptible to infections with *H. parasuis*. It has been shown that pigs infected with *M. hyopneumoniae* have more severe lesions of secondary infections with *P. multocida* (Ciprian *et al.*, 1998) or *Actinobacillus pleuropneumoniae* (Yagihashi *et al.*, 1984). To date, it remains unclear whether these combined infections also influence the transmission of *M. hyopneumoniae*.

Some considerations should be made when extrapolating the present results to field conditions. Firstly, in our experiments, pigs were free of PRRSV and were only infected with one isolate of *M. hyopneumoniae*. In commercial farms, pigs may be infected with other respiratory pathogens such as PRRSV and, although it has not yet been shown clearly, there are indications that pigs may be simultaneously infected with more than one isolate of *M. hyopneumoniae* (Stakenborg *et al.*, 2005). Secondly, the pig density in our experiment was low and optimal housing conditions were present, which may not be the case under field conditions. Thirdly, the pigs were inoculated only once and observed for a limited period of time. Under field conditions, pigs are exposed to the infection for a longer period and in most cases during the entire grow-finish period. All these arguments suggest that the experimental conditions were more ideal than what is expected in the field and therefore the estimated transmission ratios may be an underestimation of reality. Finally, it might be possible that the high experimental inoculation dose was leading to a higher shedding of *M. hyopneumoniae* by the seeder pigs, and consequently the immunity conferred by vaccination was not strong enough to prevent colonization of *M. hyopneumoniae* in the respiratory tract. The excretion level is usually not known under field conditions, but it is reasonable to assume that the excretion is lower compared with the experimental conditions. This might have led to an overestimation of the transmission ratio present under field conditions.
5. Conclusion

The present experiment showed that vaccination against *M. hyopneumoniae* with a commercial vaccine significantly reduced the clinical symptoms and lung lesions in pigs, but that only a limited and non-significant reduction in the transmission of *M. hyopneumoniae* was achieved. Although the results may not be extrapolated to field conditions as such, it was illustrated that vaccination alone with the current bacterin vaccines will likely not be sufficient to eliminate *M. hyopneumoniae* from infected pig herds.

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REFERENCES


CHAPTER 2.2.

INTERACTION OF *Mycoplasma hyopneumoniae* WITH THE RESPIRATORY TRACT
INTERACTIONS OF HIGHLY AND LOW VIRULENT *Mycoplasma hyopneumoniae* ISOLATES WITH THE RESPIRATORY TRACT OF PIGS

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**ABSTRACT**

*Mycoplasma hyopneumoniae* is the etiological agent of swine enzootic pneumonia, a chronic nonfatal disease affecting pigs of all ages. To obtain better insight in the mechanisms responsible for differences in virulence between highly and low virulent *M. hyopneumoniae* isolates, 23 caesarean-derived, colostrum-deprived piglets were randomly assigned to 3 groups. Groups 1 and 2 consisted of 9 animals each, which were intratracheally inoculated at one week of age with a highly or a low virulent isolate of *M. hyopneumoniae*, respectively. The remaining 5 animals were inoculated with sterile culture medium. Animals were euthanized at 5, 10, 15 and 28 days post inoculation (DPI). Animals inoculated with the highly virulent isolate had more neutrophils in BAL fluid at 10, 15 and 28 DPI compared to the other groups. At 10 and 15 DPI, animals in the highly virulent group had significantly higher concentrations of TNF-α in BAL fluid. IL-1β concentration in this group was higher at 5 DPI and at 28 DPI compared to the other groups. From 10 DPI onwards, significantly higher titres of *M. hyopneumoniae* were detected in the BAL fluid of animals inoculated with the highly virulent isolate compared to animals inoculated with the low virulent isolate. Additionally, the *in vitro* generation time of the highly virulent *M. hyopneumoniae* isolate was significantly shorter than that of the low virulent isolate. The present study indicates that the difference in pathogenicity between the highly and low virulent isolates is associated with a faster *in vitro* growth, a higher capacity to multiply in the lungs and the induction of a more severe inflammation process by the highly virulent isolate.
1. INTRODUCTION

*Mycoplasma hyopneumoniae* is the primary cause of enzootic pneumonia, a chronic respiratory disease in pigs. The disease is present in most intensive pig farms and is characterized by high morbidity and low mortality rates (Maes et al., 1996). The typical sign associated with a non-complicated *M. hyopneumoniae* infection is a dry and non-productive cough.

Besides management and housing conditions, the clinical outcome of a *M. hyopneumoniae* infection may also depend on the virulence of the infecting strain since major differences in virulence have been shown in field isolates. Conventional pigs were inoculated with different isolates and clinical, pathological and histopathological parameters were compared. Based on the outcomes of these experimental infections, the isolates could be classified as being highly, moderately and low virulent (Vicca et al., 2003). Although clear differences in the genomic profiles of the isolates were observed (Stakenborg et al., 2005), until now, no genetic markers associated with virulence were identified in these isolates. Also other research groups have identified antigenic (Ro and Ross, 1983) and genetic (Frey et al., 1992; Artiushin and Minion, 1996) differences between isolates of *M. hyopneumoniae*, but in these studies no information was available on the virulence of the isolates.

Lung tissue injury and disease subsequent to *M. hyopneumoniae* infections appear to be mainly caused by the host response (Thacker, 2006). The microscopic lesions associated with mycoplasmal pneumonia are indeed characterized by an infiltration in the peribronchiolar and perivascular areas with mononuclear cells, and a *M. hyopneumoniae* infection results in production of proinflammatory cytokines, including interleukin (IL)-1, IL-6, IL-8 and Tumor Necrosis Factor (TNF)-α (Asai et al., 1993, 1994; Rodriguez et al., 2004).

No information is available about the mechanisms responsible for differences in virulence between highly and low virulent *M. hyopneumoniae* isolates. As a first step to obtain better
insights in these mechanisms, the interaction of a highly and a low virulent \textit{M. hyopneumoniae} isolate with the porcine respiratory tract was studied and the inflammatory responses they induce were compared. Also the capacity of these isolates to multiply \textit{in vivo} and \textit{in vitro} was compared.
2. MATERIALS AND METHODS

2.1. Animals

Twenty-three, caesarean-derived colostrum-deprived (CDCD) piglets, from 2 sows of a herd free of *M. hyopneumoniae* and PRRS virus, were used. The piglets were individually housed in isolation units with controlled temperature and HEPA filtered air.

2.2. Challenge inocula

A highly virulent isolate (F7.2C) was obtained from a herd experiencing clinical disease associated with *M. hyopneumoniae* infections and a low virulent isolate (F13.7B) was obtained from an infected herd without clinical symptoms. The highly virulent isolate had been passed 10 times *in vitro*, while the low virulent isolate had 9 passages. Isolates were grown in modified Friis’ medium supplemented with 10% pig and 10% horse serum (*Mycoplasma* growth medium) (Friis, 1975). The isolates have been shown to be highly or low virulent during previous comparative virulence and transmission studies (Vicca et al., 2003; Meyns et al., 2004). PFGE analysis resulted in clearly different genomic patterns between both isolates (Stakenborg et al., 2005).

2.3. Experimental design

One week after birth, the piglets were inoculated intratracheally (Van Reeth *et al.*, 2000). Briefly, animals were held in vertical position with their neck extended. A needle was inserted through the skin cranial to the sternum and the inoculum was injected. A first group of 9
piglets was inoculated with 5 ml inoculum containing $5 \times 10^5$ Colour Changing Units (CCU)/ml of the highly virulent *M. hyopneumoniae* isolate F7.2C. A second group of 9 piglets was inoculated with 5 ml inoculum containing $3.5 \times 10^6$ CCU/ml of the low virulent *M. hyopneumoniae* isolate F13.7B. A third group consisted of 5 piglets that were inoculated with 5 ml *M. hyopneumoniae* growth medium (control group).

One to three piglets per group were randomly selected and euthanized at 5, 10, 15 and 28 days post inoculation (DPI) (Table 1) by deep anaesthesia with 0.3 ml/kg of a mixture of Xylazin (Xyl-M 2%, VMD, Arendonk, Belgium) and Zolazepam - Tiletamin (Zoletil® 100, Virbac, Louvain la Neuve, Belgium), followed by exsanguination.

After euthanasia, samples from the left lung were collected for histopathological and immunofluorescence (IF) examination. From the right lung, bronchoalveolar lavage (BAL) fluid was collected for bacteriological examination, cytokine measurements, cell evaluation and detection of *M. hyopneumoniae* antibodies. In addition, blood samples were taken and the sera were also analyzed for *M. hyopneumoniae* antibodies.

The study was performed after approval of the Ethical Committee for animal experiments of the Faculty of Veterinary Medicine, Ghent University.

2.4. Clinical, pathological, histopathological and immunofluorescence evaluation

Animals were observed daily to evaluate body condition, presence of dyspnea, coughing or behavioural changes. Macroscopic lesions were quantified using a lung lesion score system (Hannan *et al.*, 1982). Total lung scores could vary between score 0 (no lesions) and a theoretical maximum of 35. Tissue samples from each of the 3 left lung lobes and the accessory lobe were collected for histopathology. The samples were fixed in 10% neutral buffered formalin and routinely processed and embedded in paraffin. Using light microscopy,
peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation, were scored (score 0 - 4). Additionally, an overall score, consistent with *M. hyopneumoniae* induced pneumonia lesions (score 0 - 5) (Morris *et al*., 1995), was calculated as a mean value for the 4 lung samples. Samples of each left lung lobe were also investigated with a semi-quantitative immunofluorescence (IF) assay (score 0 - 3) to detect the presence of *M. hyopneumoniae* (Kobisch *et al*., 1978).

2.5. Bronchoalveolar Lavage Fluid, Bacteriology, Cytology and Cytokine ELISA

The right lung was used to perform bronchoalveolar lavage (BAL) in order to collect epithelial lining fluid and bronchoalveolar cells. A total of 30 ml of sterile Alsevers solution (pH 6.1) was slowly infused in the lung and aspirated back into the syringe. The recovered fluids were immediately cooled at 4°C and centrifuged at 300 g for 10 min.

The supernatant was carefully decanted from the cell pellet and immediately used for *M. hyopneumoniae* titration. This was performed on each sample of BAL fluid, by inoculating 20 µl of the recovered BAL fluid into 180 µl of *M. hyopneumoniae* culture medium. The titre was expressed as the number of CCU/ml. Standard bacteriology on BAL fluid was also performed.

The remaining fluid was stored at -80°C and thawed to 4°C when immunoassays for IL-1β, TNF-α and *M. hyopneumoniae* antibodies were performed. TNF-α and IL-1β levels in BAL fluid were measured using a commercially available immunoassay (Biosource Swine Immunoassay Kit, Biosource, Nijvel, Belgium). Samples were processed according to the manufacturers recommendations. The intensity of the coloured product was read using optical density (OD) at 450nm and quantified by the use of a standard curve, as prescribed.

The pelleted cells were washed and resuspended in phosphate buffered saline (PBS) for
cytological evaluation. The total cell count was determined by counting a fixed volume of the BAL fluid with a haemacytometer. Differential cell counts were determined by examination of 400 cells on a cytocentrifugation preparation (Cytospin 2, Shandon Southern Products Ltd., England), stained with modified Wright-Giemsa solution (Wako Pure Chemicals Industries Ltd, Osaka, Japan).

2.6. Detection of *M. hyopneumoniae* specific antibodies

Blood samples and BAL fluids taken at euthanasia from all pigs were used to detect antibodies against *M. hyopneumoniae*, using the competitive DAKO® Mh ELISA (DAKO, Glostrup, Denmark) (Feld et al., 1992). Percentage optical density (OD) was calculated as the proportion of the OD-value of the sample compared with the OD-value of a buffer control sample. Sera or BAL fluids with OD-values < 50% of the OD_{buffer-control} were considered positive. OD-values ≥ 50% of the OD_{buffer-control} were classified as negative.

2.7. *In vitro* growth rate of *M. hyopneumoniae*

The *in vitro* growth rate of both isolates was studied by determining the growth curve. Twenty-five millilitres of modified Friis medium was inoculated to a final concentration of about $10^4$ CCU/ml and incubated aerobically in a linear shaking water bath at 37°C, with a frequency of 100 rpm. Between 0 and 120 hours post inoculation, 71 (F7.2C) or 79 (F13.7B) CCU-titrations were performed for both isolates. The generation time of the *M. hyopneumoniae* isolates was calculated according to Baranyi and Roberts (1996). All data were corrected for differences in titres of both inocula, by subtracting the intercept of the predicted linear curve from each measurement for both *M. hyopneumoniae* isolates. The
experiment was carried out twice, the first time with six replicates of CCU-determinations at each time point for each isolate and the second time with four replications of CCU-determinations.

2.8. Statistical analysis

Data of bacteriology, cytokine measurements, histopathological and IF scores were analyzed using analysis of variance (ANOVA). If the P-value from the ANOVA was less than or equal to 0.05, post hoc pair wise comparisons of the different treatment groups were performed by least significant difference at $P < 0.05$ rejection level.

Data of growth curves were analyzed by a linear regression model using a log 2 transformation of the CCU-data. Assumptions for linear regression analysis, such as normality of the residuals, consistency of the residual variance and linearity, were tested each time. To verify the differences in growth rates, a general linear model (GLM) Repeated Measures Analysis was performed to assess possible differences in the course of the curves. Data were analysed using the statistical packages SPSS 12.0 (SPSS 12, SPSS Inc. Illinois, USA).
3. Results

3.1. Clinical, pathological, histological and IF observations

Results of each individual animal are presented in Table 1.
During the study, none of the animals showed any clinical sign like coughing or dyspnea. At 5 DPI, a small area of red consolidation was observed on the cardiac lobe in one animal inoculated with the highly virulent isolate. At 10 DPI, one (score 4.36) out of three animals in the highly virulent group and one (score 0.21) out of the three animals in the low virulent group, had observable lesions. Fifteen DPI, all 3 piglets in the highly virulent group had lesions (min. 0.57, and max. 10.84), while only one out of the three animals in the low virulent group had macroscopically observable lesions (score 4.64). At 28 DPI, the animal inoculated with the highly and low virulent isolate had a macroscopic score of 4.48 and 0.71, respectively. None of the animals of the negative control group had any lesions. No statistically significant differences were observed between the groups.

At 15 and 28 DPI, overall histological lesions consistent with *M. hyopneumoniae* infections were significantly higher (*P* < 0.05) in animals inoculated with the highly virulent isolate (score between 2.25 - 3.75), compared with animals of the low virulent group (score between 0.75 - 1.75) and the control group (between 0.50 - 0.75).

IF revealed the presence of *M. hyopneumoniae* on the epithelium lining of bronchi and bronchioli of pigs inoculated with the highly or low virulent isolate. No IF staining was observed in the negative control animals. At 5 and 10 DPI, the IF score of animals inoculated with the highly virulent isolate was only numerically higher compared to the animals inoculated with the low virulent isolate. At 15 DPI, a significantly higher (*P* < 0.05) IF score
Table 1: Summary of the results of 23 CDCD piglets inoculated with a highly or a low virulent M. hyopneumoniae isolate or with culture blank medium. Piglets were euthanized at different days post inoculation and different parameters were investigated.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>DPI</th>
<th>Animal number</th>
<th>Lung lesion score (0-35)</th>
<th>IF score (0-3)</th>
<th>Histology score (0-5)</th>
<th>Cuffing score (0-4)</th>
<th>Time of M. hyopneumoniae (CCU/ml) in BAL fluid</th>
<th>Number of cells/ml BAL fluid (x10^7)</th>
<th>% of Macrophages in BAL fluid</th>
<th>% of Neutrophils in BAL fluid</th>
<th>% of Lymphocytes in BAL fluid</th>
<th>TNF-α (pg/ml) BAL fluid</th>
<th>IL-1β (pg/ml) BAL fluid</th>
<th>% OD in DAKO ELISA on serum</th>
<th>% OD in DAKO ELISA on BAL fluid</th>
</tr>
</thead>
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<tr>
<td>Highly Virulent isolate</td>
<td>5</td>
<td>1</td>
<td>0.00</td>
<td>0.33</td>
<td>1.00</td>
<td>0.75</td>
<td>5x10^5</td>
<td>1.08</td>
<td>88.5</td>
<td>1.0</td>
<td>10.6</td>
<td>22.8</td>
<td>106.9</td>
<td>104</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.42</td>
<td>0.33</td>
<td>0.25</td>
<td>0.00</td>
<td>5x10^5</td>
<td>3.13</td>
<td>93.6</td>
<td>0.4</td>
<td>6.0</td>
<td>18.6</td>
<td>153.4</td>
<td>111</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.00</td>
<td>0.33</td>
<td>0.75</td>
<td>0.00</td>
<td>5x10^5</td>
<td>1.40</td>
<td>76.6</td>
<td>0.0</td>
<td>23.4</td>
<td>31.6</td>
<td>81.5</td>
<td>75</td>
<td>104</td>
<td></td>
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<tr>
<td>Low virulent isolate</td>
<td>10</td>
<td>4</td>
<td>0.00</td>
<td>0.33</td>
<td>0.75</td>
<td>0.50</td>
<td>5x10^5</td>
<td>2.00</td>
<td>72.1</td>
<td>10.5</td>
<td>17.4</td>
<td>66.6</td>
<td>102.6</td>
<td>59</td>
<td>107</td>
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<tr>
<td></td>
<td>5</td>
<td>4.36</td>
<td>0.67</td>
<td>2.25</td>
<td>1.50</td>
<td>2.75x10^5</td>
<td>3.88</td>
<td>79.3</td>
<td>3.4</td>
<td>17.3</td>
<td>205.4</td>
<td>50.5</td>
<td>90</td>
<td>99</td>
<td></td>
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<tr>
<td></td>
<td>15</td>
<td>7</td>
<td>10.84</td>
<td>2.00</td>
<td>3.75</td>
<td>2.50</td>
<td>5x10^6</td>
<td>1.90</td>
<td>51.1</td>
<td>8.0</td>
<td>40.9</td>
<td>124.0</td>
<td>102.6</td>
<td>37</td>
<td>65</td>
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<td>Culture blank medium</td>
<td>28</td>
<td>9</td>
<td>4.48</td>
<td>2.00</td>
<td>2.75</td>
<td>2.50</td>
<td>5x10^6</td>
<td>27.50</td>
<td>48.3</td>
<td>24.9</td>
<td>26.7</td>
<td>335.3</td>
<td>1089.5</td>
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<tr>
<td></td>
<td>5</td>
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<td>0.00</td>
<td>1.00</td>
<td>0.75</td>
<td>2.75x10^4</td>
<td>0.80</td>
<td>91.7</td>
<td>0.0</td>
<td>8.3</td>
<td>31.1</td>
<td>53.4</td>
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<td>117</td>
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<tr>
<td></td>
<td>11</td>
<td>0.00</td>
<td>0.33</td>
<td>0.50</td>
<td>0.25</td>
<td>2.75x10^4</td>
<td>1.78</td>
<td>89.7</td>
<td>0.0</td>
<td>10.3</td>
<td>22.2</td>
<td>74.5</td>
<td>101</td>
<td>111</td>
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<tr>
<td></td>
<td>12</td>
<td>0.21</td>
<td>0.33</td>
<td>1.00</td>
<td>0.50</td>
<td>5x10^5</td>
<td>0.73</td>
<td>82.1</td>
<td>0.0</td>
<td>17.9</td>
<td>22.2</td>
<td>74.5</td>
<td>101</td>
<td>111</td>
<td></td>
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<tr>
<td></td>
<td>13</td>
<td>0.00</td>
<td>0.33</td>
<td>1.50</td>
<td>0.50</td>
<td>5x10^5</td>
<td>0.88</td>
<td>77.3</td>
<td>1.2</td>
<td>21.5</td>
<td>26.4</td>
<td>73.1</td>
<td>72</td>
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<tr>
<td>Low virulent isolate</td>
<td>14</td>
<td>0.00</td>
<td>0.00</td>
<td>1.00</td>
<td>0.25</td>
<td>5x10^5</td>
<td>1.20</td>
<td>70.1</td>
<td>0.0</td>
<td>29.9</td>
<td>57.7</td>
<td>49.1</td>
<td>51</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>Culture blank medium</td>
<td>28</td>
<td>18</td>
<td>0.71</td>
<td>1.00</td>
<td>1.50</td>
<td>0.75</td>
<td>5x10^5</td>
<td>4.70</td>
<td>69.6</td>
<td>2.0</td>
<td>28.4</td>
<td>89.9</td>
<td>30.8</td>
<td>14</td>
<td>54</td>
</tr>
</tbody>
</table>

1 Days Post Inoculation; 2 Immunofluorescence; 3 Colour Changing Units; 4 Bronchoalveolar lavage; 5 Optical Density (sample / positive control)
was observed in animals inoculated with the highly virulent isolate compared to the animals of the low virulent group.

3.2. Bacteriology and cytology of BAL fluid

Results of titrations of *M. hyopneumoniae* in the lungs of inoculated pigs, expressed as CCU/ml, are shown in Table 1. Significantly higher numbers of *M. hyopneumoniae* organisms were cultivated from the BAL fluid of animals in the highly virulent group at 10 and at 15 DPI, compared with animals of the low virulent group. Standard bacteriological culture remained negative for all piglets.

No major differences were observed in the total number of cells and the percentage of macrophages and lymphocytes present in the BAL fluid sample of the different groups except for pig nr. 9 inoculated with the highly virulent isolate, which had clearly more cells and a higher percentage of neutrophils in the BAL fluid (Table 1). From 10 DPI onwards, the percentage of neutrophils was, however, significantly higher in animals inoculated with the highly virulent isolate.

3.3. Cytokines in BAL fluid

For TNF-α, at 5 DPI, no significant differences were found between *M. hyopneumoniae* inoculated and control animals. From 10 DPI onwards, significantly higher concentrations were found in animals from the highly virulent group compared with the control animals. TNF-α concentration in BAL fluid of animals from the low virulent group was intermediate between both other groups. Higher concentrations of TNF-α in BAL fluid tended to be associated with more severe pathological scores.
For IL-1β, at 5 DPI, a higher concentration was observed in the highly virulent group, while at 10 and 15 DPI, no major differences were observed. At 28 DPI, a very high concentration of IL-1β was found in the animal inoculated with the highly virulent isolate.

3.4. Detection of *M. hyopneumoniae* specific antibodies

In sera, at 5 and 10 DPI, no animals showed positive results, but sera from 10 DPI, tended to have lower OD-values in animals inoculated with *M. hyopneumoniae* compared to control animals. At 15 DPI, two out of the three animals in both the highly and low virulent group were positive for serum antibodies. At 28 DPI, both *M. hyopneumoniae* inoculated animals were positive. All non-inoculated animals were negative. Animals with lowest OD-values tended to have macroscopically more severe lesions compared to animals with higher OD-values.

In BAL fluid, at 15 DPI, the animals with the highest macroscopic lung lesion score, had the lowest OD-value. At 10 and 15 DPI, mean OD-values of animals of the highly virulent group were lower, but not significantly ($P = 0.09$) compared with animals of the low virulent group. Only one animal inoculated, with the highly virulent was positive for antibodies in the BAL fluid, at 28 DPI, while all other animals were negative.

3.5. *In vitro* growth rate of *M. hyopneumoniae*

The *in vitro* growth curve of the highly and the low virulent isolates is presented in Fig 1. This curve presents the mean values of all measurements on each time point. The *in vitro* generation time was 6h 36min for the highly virulent isolate and 8h 46min for the low virulent isolate ($P < 0.05$).
Fig 1. *In vitro* growth curves of highly (●) and low (□) virulent *M. hyopneumoniae* isolates.
4. DISCUSSION

The multiplication of *M. hyopneumoniae* in the lungs of pigs results in pneumonia characterized by cellular infiltration of macrophages, neutrophils and lymphocytes (Blanchard *et al.*, 1992). Although distinct variations between individual animals were observed, macroscopic lesions in the highly virulent group tended to be more pronounced. Additionally, the highly virulent group showed higher histological cuffing scores at 15 and 28 DPI. From 10 DPI onwards, more neutrophils were observed in the BAL fluid of animals of the highly virulent group. These results confirm the difference in virulence between the highly and the low virulent isolates, which was previously demonstrated in conventional animals (Vicca *et al.*, 2003; Meyns *et al.*, 2004). Additionally, the study demonstrated that the highly virulent isolate had a capacity to induce lesions and inflammatory processes earlier after inoculation, compared to the low virulent isolate.

Caesarean-derived colostrum-deprived piglets, grown in sterile conditions, were used in this experimental study because these animals provide a unique possibility to investigate the interaction of *M. hyopneumoniae* with the respiratory tract without confounding effects of other pathogens. A disadvantage of the use of gnotobiotic piglets is the limited number of animals that can be used.

One of the first steps in the pathogenesis of *M. hyopneumoniae* infections is the adherence of the agent to the respiratory tract (Zielinski and Ross, 1992). In the present studies, IF staining revealed the presence of *M. hyopneumoniae* on epithelium lining the bronchi and bronchioli of pigs inoculated with the highly and low virulent isolate, indicating that both isolates were able to adhere to epithelial cells of the respiratory tract. In previous studies, no clear differences were observed in adhesion capacity of both isolates to porcine tracheal epithelial cells *in vitro* (non-published results). The number of reiterated region RR1 tandem
repeats of the P97 adhesions genes was 15 and 13 for the highly and the low virulent isolate, respectively (Stakenborg et al., 2006). At least seven RR1 repeats seem to be necessary to allow a strain to adhere to porcine tracheal cells in vitro (Hsu and Minion, 1998). Taking these observations together, it can be concluded that there are no clear indications that differences in virulence between the *M. hyopneumoniae* isolates used in the present studies are due to differences in adherence capacity between both isolates.

In animals of the highly virulent group, a higher titre of *M. hyopneumoniae* organisms was found in the BAL fluid at 10 and 15 DPI compared with the titre in BAL fluid of animals of the low virulent group. In previous studies, where animals were inoculated with the same highly or low virulent *M. hyopneumoniae* isolate, the more intense IF-staining also indicated in a semi quantitative way, that higher numbers of *M. hyopneumoniae* organisms were present in the lungs of animals infected with the highly virulent isolate at 28 or 42 DPI (Vicca et al., 2003, Meyns et al., 2004). Based on the results of the present study, the capacity of the isolates to multiply in the respiratory tract seems to be correlated with the severity of the lung lesions. Different mechanisms can be assumed to explain differences in capacity to multiply in vivo. Firstly, it is possible that the highly virulent isolate has an intrinsic faster growth rate. This is supported by the significantly shorter in vitro generation time of the highly virulent isolate (F7.2C) compared with the low virulent isolate (F13.7B). Although parallel findings were found between the in vivo and in vitro growth rate of the isolates, more isolates should be tested, both in vivo and in vitro, to confirm these results and to make general conclusions. Secondly, it is possible that the growth of the low virulent isolate is more inhibited by host defence mechanisms in the respiratory tract. Indeed, although similar *M. hyopneumoniae* titres were detected in the BAL fluid of the highly and the low virulent groups at 5 DPI, at 10 and 15 DPI, the titre of *M. hyopneumoniae* was significantly higher in the BAL fluids of the highly virulent group, indicating a more efficient inhibition of the low virulent isolate. Most
probably, antibodies do not play a major role in this possible inhibitory effect since OD-values measured with the blocking ELISA were lower in the serum and BAL fluid of animals inoculated with the highly virulent isolate.

The multiplication of *M. hyopneumoniae* in the lungs results in inflammation characterized by cellular infiltrations of macrophages, neutrophils and lymphocytes (Blanchard *et al.*, 1992). It has been suggested that alveolar macrophages have a prominent role in the initiation of an inflammatory response starting shortly after infection, since these cells were infected with *M. hyopneumoniae* in the early stage of disease and readily expressed IL-1 and TNF-α in response to infection (Choi *et al.*, 2006). TNF-α is chemotactic for of neutrophils (Smart and Casale, 1994). In our study, animals of the highly virulent group had a significantly higher proportion of neutrophils in their BAL fluid from 10 DPI onwards, and a higher cuffing score from 15 DPI onwards, compared with animals of the low virulent group. Additionally, the level of TNF-α in the BAL fluid was also higher in animals inoculated with the highly virulent isolate. This indicates that inflammatory cell responses in the lungs are more pronounced in case of infections with the highly virulent isolate. It is known that low doses of TNF-α result in the stimulation of mononuclear phagocytes and a polyclonal activation of B cells, while in higher concentrations, TNF-α causes tissue injury and pathology. In animals inoculated with the highly or the low virulent isolate, higher IL-1β concentrations were observed at form 5 DPI onwards compared to non inoculated animals, which is in accordance with other studies (Asai *et al.*, 1993, Rodriguez *et al.*, 2004). The higher IL-1β concentration at 5 DPI and at 28 DPI in the animals of the highly virulent group compared to the concentration in animals of the low virulent group indicates that the highly virulent isolate induces a faster onset of the inflammation process and a more intense inflammation at 28 DPI. Although the inflammation process may be important to suppress micro-organisms infecting the lungs, it seems to be less effective against the highly virulent *M. hyopneumoniae* isolate.
since higher *M. hyopneumoniae* titres were detected in the lungs of animals inoculated with the highly virulent isolate.

It is not clear which *M. hyopneumoniae* antigens are involved in the inflammatory process, but lipoproteins are good candidates. A macrophage activating lipoprotein (MALP-2), integrated in the plasma membrane of *Mycoplasma fermentans*, has already been characterized (Mühlradt *et al.*, 1997; Deiters and Mühlradt, 1999, Lührmann *et al.*, 2002). This lipoprotein is recognized by Toll-like receptors (TLR)2 and TLR6 (Akira and Hemmi, 2003; Into *et al.*, 2004). MALP-2 like structures have been found in other mycoplasmas as well, including *M. arthritidis* (Cole, 1991), *M. hyorhinis* (Mühlrath *et al.*, 1998) and *M. salivarum* (Okusawa *et al.*, 2004). Several lipoproteins have been described in *M. hyopneumoniae* (Wise and Kim, 1987) and the recently sequenced genome of *M. hyopneumoniae* revealed 53 open reading frames with prokaryotic lipoprotein lipid attachment sites (Minion *et al.*, 2004). It remains to be determined if some of these play a role in TNF-α induction by porcine macrophages and if there are differences between highly and low virulent *M. hyopneumoniae* isolates.

In conclusion, our results demonstrate that the highly virulent *M. hyopneumoniae* isolate used in this study had a higher capacity to multiply in the lungs of infected pigs, multiplied faster *in vitro* and induced a more severe inflammation process than the low virulent isolate.
ACKNOWLEDGEMENTS

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REFERENCES


CHAPTER 3.

GENERAL DISCUSSION
GENERAL DISCUSSION

*M. hyopneumoniae* infections are responsible for respiratory disease in pigs. Although the infection is very common, the clinical outcome of the disease is highly variable and influenced by environmental factors, management practices on the pig farm (Maes et al., 1996) and by the virulence of the strains present in the farm (Vicca et al., 2003). Apart from the factors that influence the clinical outcome of the infection, little was known about the infection dynamics (shedding and transmission) of *M. hyopneumoniae* in pig populations. The present research was performed to gain better insight in the transmission of *M. hyopneumoniae* during the nursery period and to investigate possible differences in transmission between highly and low virulent isolates. Further, the designed transmission model was also used to evaluate the effect of vaccination on the transmission of the organism. Additionally, bacteria-host interactions of highly and low virulent isolates of *M. hyopneumoniae* were compared and mechanisms by which the organism induces lesions in piglets were investigated.

Since *M. hyopneumoniae* plays a key role in the PRDC complex, it is important to investigate the transmission of the organism in nursery piglets and to define possible strategies to prevent infections during this period. Although clinical symptoms of mycoplasmal pneumonia are not common before 6 weeks of age (Ross, 1999), it is believed that infection is already present at the start of the nursery period in a low number of animals (Calsamiglia and Pijoan, 2000; Vicca et al., 2002). In the nursery room, weaned piglets from different sows are placed together for the first time, which represents an important stress factor. Therefore, at that time, animals are likely to be more susceptible to various infectious diseases. To assess the transmission rate of *M. hyopneumoniae* during the nursery period, an
adjusted reproduction ratio ($R_n$) was used. This $R_n$-value differs from the conventional Basic Reproduction Ratio ($R_0$) because the transmission was quantified during this strictly defined observation period and not during the entire infectious period of the animal as is the case for $R_0$. As a consequence, the calculated reproduction ratio is only valid for this specified period. The entire infectious period could not be taken into account because it can last up to more than 6 months and it is highly variable for *M. hyopneumoniae* (Fano et al., 2005).

The $R_n$-values were calculated by the use of the final size at the end of the observation period. By measuring the infectious state of all animals at the end of the experiment, it was possible to calculate the $R_n$-value with the maximal likelihood estimator and to compare this value in different experimental settings. This method provides the $R_n$ with the highest probability to fit with the outcomes observed in the transmission experiments. A prerequisite for this method is the availability of a diagnostic method that accurately assesses the infectious state of an animal at the end of the experiment. In our studies, nPCR on BAL fluid, obtained from euthanized animals at the end of the observation period was used for this purpose. When the transmission rate is calculated by the use of the Generalized Linear Model or the Martingale estimator, information is needed about the infectious state of the animals during the experiment. The advantage of the use of the GLM method is that the infection rate $\beta$ can be calculated separately from the $R_0$-value. The Martingale estimator is more suitable for the estimation of $R_0$ in infinite large populations (e.g. Wildlife epidemiology). By sampling live animals during an experiment with *M. hyopneumoniae*, only scarce information can be achieved about their true infectious state. Serology is not a reliable tool, since individually infected animals do not have a uniform serologic response to infection, especially when a low infection level is present (Leon et al., 2001). Additionally, a considerable variation between the timing of infection and the occurrence of seroconversion can be present.
(Sørensen et al., 1997). Nasal swabs investigated with nPCR are not suited for an accurate determination of the onset of the infectious period. For these samples, the only certainty in case of a positive result is the fact that DNA from live or dead *M. hyopneumoniae* organisms is present in the swab. Additionally, animals which harbor the organism in the nose are not necessarily infected in their lower airways and will not always be infectious to other animals (Kurth et al., 2002). BAL fluid may also be obtained from live animals, but Marois et al. (2007) showed that several BAL samplings performed on the same pig influence the lung lesion extent and may have an effect on the host defense mechanisms. By consequence, this technique was believed to be too invasive on the animals and therefore it might interfere with transmission of the pathogen. It can be concluded that, at present, no diagnostic tool is available for an accurate determination of the infectious state of animals during a transmission experiment with *M. hyopneumoniae*. Therefore, the maximal likelihood estimator which only requires measuring the infectious state at the end of the experiment can be considered as the only available method to calculate the $R_{0}$-value for *M. hyopneumoniae* in transmission experiments.

In chapter 2.1.1., the transmission model was successfully used to evaluate the transmission of highly (F7.2C) and low (F13.7B) virulent isolates of *M. hyopneumoniae*. Since no significant difference was observed between these isolates, an overall $R_{0}$-value of 1.16 (0.94 - 4.08) was calculated. This means that one piglet will infect on average one susceptible penmate during the nursery phase. The results of this study confirm field observations indicating that *M. hyopneumoniae* organisms spread relatively slowly through infected herds (Leon et al., 2001). It has to be kept in mind that, when transmission rates are used, the use of confidence intervals is more informative than the point estimates of the $R_{0}$-values, since small differences in the outcomes of an experiment can have important
influences on the point estimate. In our situation, this means that, with a 95% confidence interval, the transmission of \( M. \) hyopneumoniae during the nursery phase, independently of the isolate, is between 0.94 and 4.08. This can be considered as a general number, applicable to many \( M. \) hyopneumoniae field isolates. Consequently, each animal infected before weaning, will infect between one and four other piglets during a nursery period of 6 weeks. The threshold theorem, which states that endemic infections fade out when the \( R_0 < 1 \), can not be used for the evaluation of the \( R_0 \)-value. For this purpose, the \( R_0 \) of \( M. \) hyopneumoniae should be calculated taking into account the entire infectious period of an infected animal (farrowing + nursery + fattening period). Since \( R_0 \) is already larger than one, the \( R_0 \) of \( M. \) hyopneumoniae will also be larger than one under the present conditions.

Although no significant difference was observed in the transmission of the highly (F7.2C) and the low virulent isolates (F13.7B), the transmission ratio of the highly virulent isolate (\( R_n = 1.47 \) (0.68 - 5.38)) was slightly higher compared to the \( R_n \)-value of the low virulent isolate (\( R_n = 0.85 \) (0.33 - 3.39)). Since only a limited number of 24 animals were used to estimate the transmission rate of each isolate, the power of the study to significantly differentiate the reproduction ratios was rather low. Using more animals could have resulted in a significant difference. Additionally, the faster occurrence of seroconversion after inoculation and the more intense IF scoring, pointed to a more effective multiplication of the highly virulent \( M. \) hyopneumoniae isolate in the lungs of inoculated pigs. In Chapter 2.2., the hypothesis of a faster multiplication of the highly virulent isolate in the respiratory tract of gnotobiotic piglets was confirmed, since higher titres of \( M. \) hyopneumoniae organisms were present in the BAL fluid of gnotobiotic piglets inoculated with the highly virulent isolate from 10 DPI onwards compared to the BAL fluids of animals inoculated with the low virulent isolate. This was observed despite the use of a slightly lower inoculation dose in the highly virulent group (5 ml
inoculum containing \(5 \times 10^5\) (CCU/ml) compared to the inoculation dose (5 ml inoculum containing \(3.5 \times 10^6\) CCU/ml) for the low virulent group. The higher multiplication of the highly virulent isolate may result in a higher excretion of this isolate by inoculated animals, but probably, the difference in excretion was too small to lead to significantly different infection rates in the current experimental setting.

The second study (Chapter 2.1.2.) showed that vaccination with a currently available vaccine was not able to prevent transmission of \(M.\ hyopneumoniae\). This finding confirms what has been observed in several field studies, namely that the number of vaccinated seropositive pigs gradually increases towards the end of the finishing period, indicating that \(M.\ hyopneumoniae\) can still circulate in vaccinated pigs (Le Grand and Kobisch, 1996; Maes et al., 1999). When comparing the \(R_n\) in the unvaccinated (3.51) and the vaccinated groups (2.38), a numerical but not significant reduction in transmission was observed. At present, no accurate diagnostic tools are available for quantifying the bacterial load of \(M.\ hyopneumoniae\) in the BAL fluid in conventional animals. The less intense IF scores and the lower number of nPCR positive nasal samples in the inoculated and vaccinated animals compared to the unvaccinated animals, however suggest that the number of \(M.\ hyopneumoniae\) organisms in the lungs of the vaccinated animals was lower and probably resulted in a slightly lower excretion. Likely, vaccination may decrease the number of organisms, but is not able to prevent colonization or transmission of the organism. Since all currently used \(M.\ hyopneumoniae\) vaccines are bacterins, it is reasonable to assume that none of these vaccines is able to prevent colonization and transmission of the infection.

Since eradication of \(M.\ hyopneumoniae\) is not possible by the use of the currently inactivated vaccines alone, other measures should additionally be taken. Transmission of \(M.\)}
hyopneumoniae from infected sows to their offspring can be prevented by the use of medication (Alexander et al., 1980). When eradication of M. hyopneumoniae at herd level is aimed by vaccination alone, a new generation of vaccines will be needed. Such vaccines should induce a decreased infectivity of infectious animals and/or induce resistance of susceptible animals to that extent where $R_0$ of M. hyopneumoniae becomes lower than one. The development of these vaccines may be based on the identification of specific mechanisms involved in the colonization of the organism in the respiratory tract. Knowledge of these mechanisms may help to design vaccines inducing immune mechanisms that interfere with colonization.

When the transmission rates of the highly virulent isolate (F7.2C) in the first experiment ($R_n = 1.47 (0.68 - 5.38)$) are compared with the $R_n$ of the same isolate in the non-vaccinated groups of the second experiment ($R_n = 3.51 (1.51 - 9.34)$), the observed difference seems important at the first sight. Estimation of transmission parameters is, however, based upon a relative low number of animals. So, small differences in the outcome of the experiment can have important influences on the estimation of the transmission rate. The second estimate falls within the confidence limits of the first estimate. The results also confirm that, due to biological variation, the results of transmission experiments performed under highly comparable, but not identical conditions seldomly provide the same point estimate of the $R_n$-value. The difference is most probably due to a faster onset of the transmission in the second experiment. Seroconversion was already present at 29 DPI, while in the first experiment, seroconversion was only detected for the first time at 43 DPI. The first experiment was performed in spring, while the second experiment was performed during fall, with a possibly higher humidity of the air and more favorable circumstances for the survival and the transmission of the organism. Animals were purchased from the same farm, and they had a
comparable genetic background. So, possible differences in susceptibility are unlikely but cannot be totally ruled out. In conclusion, the results from different experiments should be compared with care, and conclusions are only valid if two treatments are directly compared in the same trial.

The results of the transmission experiments cannot be extrapolated to field situations as such. In the current transmission experiments (Chapter 2.1), very high doses were used to inoculate the animals. Although infection doses are not known under field conditions, it is reasonable to believe that concentrations of $10^7$ CCU per ml are not reached in the field. 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 and air quality in the experimental setting were probably better than those present under field conditions. Also, infections with pathogens like PRRSV or *A. pleuropneumoniae* were not present in our experimental animals. These infections can influence the clinical outcome of *M. hyopneumoniae* infections (Yagihashi et al., 1984; Thacker et al., 1999) and may also influence the transmission of *M. hyopneumoniae*. Considering these arguments, one may conclude that the result of the experiment can be either an over- or an under-estimation of the transmission under field conditions. Although extrapolation of results obtained in an experimental model to the field situation should be done with caution, the model developed here is useful to estimate the effects of treatment and control measures on the spread of *M. hyopneumoniae* during the nursery period. In this case $R_0$-values in treated and non-treated groups should be compared. If future eradication programs are designed, they can be evaluated in an experimental setting before they are used.
on a large scale, like it was performed previously for the eradication programs of Aujeszky’s disease (Bouma et al., 1996).

Different strains of *M. hyopneumoniae* can be present at the same time in one farm (Stakenborg, 2005), but it is not known if this influences the transmission patterns in a herd. Induction of cross-protection between different strains should be evaluated. It should also be determined if some strains have a higher capacity to persist and to spread in the herd compared to others. Such experiments may include identification of different *M. hyopneumoniae* strains in one herd at different time points. Also the behavior of newly introduced strains in farms should be studied. In order to carry out these experiments, fast and reliable techniques allowing direct detection of different *M. hyopneumoniae* strains in samples of infected animals without the requirement of isolation of this fastidious micro-organism are needed.

In the experiment described in chapter 2.2., the highly virulent strain and the low virulent strain were inoculated in mycoplasmal growth medium and the *in vitro* growth was followed by CCU-titrations. These studies indicated a faster *in vitro* multiplication of the highly virulent isolate during the logarithmic growth phase. However, this was not confirmed in studies carried out later with several highly and low virulent isolates. In these studies, it became obvious that the reproducibility of CCU-titrations was rather low. Measurement of ATP production as determined by light production in an enzymatic luciferine/luciferase reaction has a higher reproducibility (Chapelle and Levin, 1968). Since the ATP production of a *M. hyopneumoniae* cell is constant during the logarithmic growth phase, the ATP concentration is correlated with the number of organisms present in the culture. Using this assay, it was found that strains of varying virulence had a generation time of about 7 hours.
Consequently, the in vitro growth rate cannot be used as an indication of the virulence of the organism (Calus et al., unpublished results). Further research is going on to compare the colonization characteristics of highly and low virulent isolates.

In chapter 2.2., it was shown that the severity of the lesions is concurrent with the production of TNF-α in the BAL fluid. This is in agreement with the study of Asai et al. (1993). Van Reeth et al. (1999) demonstrated that an infection with PRRSV results in increased IL-1 production, whereas increases in TNF-α are negligible. The combination of the induction of TNF-α by M. hyopneumoniae and IL-1 by PRRSV in the acute stage of a combined infection may explain the more severe lesions observed after infection with both pathogens (Thacker et al., 1999). These severe respiratory problems are also observed in herds affected by PRDC, where PRRSV and M. hyopneumoniae infections are often present at the same time.

A remarkable increase in the number of neutrophils in the BAL fluid of animals inoculated with the highly virulent isolate was observed (chapter 2.2.). This is in accordance with the observation that TNF-α is chemotactic for neutrophils (Smart and Casale, 1994). Neutrophils may contribute to lesion development by production of oxygen radicals and proteolytic enzymes. Myeloperoxidase released by these phagocytes converts the relatively less toxic hydrogen peroxide produced by macrophages and neutrophils into hypochlorous acid, the most potent cytotoxic oxidant generated during neutrophilic inflammations (Sibille and Reynolds, 1990).

Differences in virulence between M. hyopneumoniae isolates may also be explained by differences to avoid host clearance mechanisms. Indeed at 5 DPI, highly and low virulent
isolates reached around the same titre in BAL fluid, while at 10 and 15 DPI, titres of the low virulent isolate were clearly lower. This finding can indicate a more efficient inhibition of the low virulent isolate. Most probably, antibodies do not play a major role in this possible inhibitory effect since a higher titre of antibodies was present in the BAL fluid of animals inoculated with the highly virulent isolate compared with the low virulent isolate.

In conclusion, in this thesis it was shown that a highly virulent isolate had a slightly, but not significantly, higher capacity to spread during the nursery period, had a higher capacity to multiply in the lungs and induced a more severe inflammatory process. Further studies are necessary to identify specific virulence factors responsible for these findings. It also appeared that vaccination with the current vaccines is not able to sufficiently reduce the transmission of *M. hyopneumoniae* to achieve eradication. The transmission model can be used to evaluate the impact of treatment and control measures on the transmission of the organism.
References


SUMMARY - SAMENVATTING
**SUMMARY**

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the primary agent of enzootic pneumonia and it also plays a major role in the Porcine Respiratory Disease Complex (PRDC) in pigs. In single infections, *M. hyopneumoniae* is relatively benign, but in field situations, the organism acts as a door opener for complicated respiratory diseases. The high and worldwide prevalence, together with the significant and long lasting economic losses contributes to the fact that *M. hyopneumoniae* is one of the most important pathogens in modern swine industry.

Eradication of the organism at farm level has been described, but reinfections are frequently observed, mainly due to the import of subclinically infected animals or through airborne transmission by infected neighbourhood farms. In the farm, the pathogen is mainly transmitted through nose-to-nose contact or by close contact. Young animals can be infected by the sow, while further spread of the infection occurs during the nursery period. By the end of the finishing period, a high percentage of the animals is usually infected. At present, control of the disease is mainly achieved by optimisation of management and housing conditions, antimicrobial medication, and vaccination, but the impact of these measures on the transmission of the organism is not well investigated.

Although epidemiological knowledge and descriptions of transmission patterns of *M. hyopneumoniae* infections have extensively been published, the transmission of *M. hyopneumoniae* was never evaluated and quantified in an experimental setting. Knowledge about the importance of the transmission of *M. hyopneumoniae* in young piglets and insight in factors that can influence this transmission are key points in controlling the disease.

Recently, isolates of *M. hyopneumoniae* with highly divergent virulence have been described, and until present, no information is available about possible differences in the interaction of different *M. hyopneumoniae* isolates with the porcine respiratory tract.
The aims of the present thesis were to obtain better insights in the transmission of *M. hyopneumoniae* during the nursery period by the creation of an experimental transmission model. Possible differences in transmission of highly and low virulent isolates were investigated. The model was used to evaluate the effect of vaccination on the transmission of the organism. Additionally, bacteria-host interactions of highly and low virulent isolates of *M. hyopneumoniae* were compared and mechanisms by which the organism induces lesions in piglets were investigated.

In the first study (Chapter 2.1.1.), the transmission of two different *M. hyopneumoniae*-isolates was investigated and quantified under experimental conditions by means of an adjusted reproduction ratio ($R_n$). This $R_n$-value, calculated according to the final size method, expresses the mean number of secondary infections due to one typical infectious piglet during the nursery period. This period lasts from 4 to 10 weeks of age, and corresponds with the nursery period used in most European production systems. Additionally, a comparison was made between transmissions of highly virulent and low virulent isolates.

Forty-eight weaned piglets, free of *M. hyopneumoniae*, were housed in 6 separate pens. During 6 weeks, 2 animals experimentally infected with *M. hyopneumoniae* were housed together with 6 susceptible piglets. At the end of the study, the number of contact-infected animals was determined by the use of nPCR on bronchoalveolar lavage (BAL) fluid. The $R_n$-values of the highly virulent and the low virulent isolates were estimated to be 1.47 (0.68 - 5.38) and 0.85 (0.33 - 3.39), respectively. No significant difference between the groups was found ($P = 0.53$). The overall $R_n$ was estimated to be 1.16 (0.94 - 4.08). It was concluded that, under the actual experimental conditions, the transmission rate of *M. hyopneumoniae*, assessed for the first time by a reproduction ratio, showed that one piglet, infected before weaning, will infect between one and four penmates during the nursery phase.
Vaccination is regularly used in an attempt to control the disease, but the influence of vaccination on the transmission was not yet evaluated. In chapter 2.1.2., a transmission experiment was performed to quantify the effect of vaccination on the transmission of *M. hyopneumoniae* in nursery piglets by means of an adjusted reproduction ratio. Thirty piglets, vaccinated at 1 week of age, and 30 non-vaccinated piglets, free of *M. hyopneumoniae*, were housed in 6 separate pens. In each pen, three animals, intratracheally inoculated with *M. hyopneumoniae*, were housed together with 7 susceptible, contact-piglets. The transmission was studied during the conventional nursery period of 6 weeks. At the end of the study, the infectious status of the animals was determined based on the results of a nPCR test, performed on BAL fluid. The $R_n$-value in the vaccinated group was 2.38 (1.07 - 7.53) while in the non-vaccinated group, an $R_n$-value of 3.51 (1.51 - 9.34) was observed, both not significantly different from each other ($P = 0.77$). Under the actual experimental conditions, transmission of *M. hyopneumoniae* in nursery piglets was only numerically lower in vaccinated groups. In addition, vaccination with a conventional vaccine could not prevent the establishment of *M. hyopneumoniae* organisms in the lung.

In chapter 2.2., the interaction of a highly virulent and a low virulent isolate of *M. hyopneumoniae* with the porcine respiratory tract was studied. To obtain better insights in the mechanisms responsible for differences in virulence between these *M. hyopneumoniae* isolates, 23 caesarean section-derived, colostrum-deprived piglets were randomly assigned to 3 groups. Groups 1 and 2 consisted of 9 animals each, which were intratracheally inoculated at 1 week of age with a highly or a low virulent isolate of *M. hyopneumoniae*, respectively. The remaining 5 animals were inoculated with sterile culture medium. Animals were euthanized at 5, 10, 15 and 28 days post inoculation (DPI). Animals inoculated with the highly virulent isolate had more neutrophils in BAL fluid at 10, 15 and 28 DPI compared to the other groups. At 10 and 15 DPI, animals in the highly virulent group had significantly
higher concentrations of TNF-α in BAL fluid. The IL-1 concentration in this group was higher at 5 DPI and at 28 DPI compared to the other groups. From 10 DPI onwards, significantly higher titres of *M. hyopneumoniae* were detected in the BAL fluid of animals inoculated with the highly virulent isolate compared to animals inoculated with the low virulent isolate. Additionally, the *in vitro* generation time of the highly virulent *M. hyopneumoniae* isolate was significantly shorter than that of the low virulent isolate. The study indicated that the difference in pathogenicity between the highly and low virulent isolates was associated with a higher capacity to multiply in the lungs and a faster induction of a more severe inflammation process by the highly virulent isolate.

From the studies described in this thesis, it can be concluded that *M. hyopneumoniae* spreads relatively slowly during the nursery period. A highly virulent isolate had a slightly, but not significant, higher capacity to spread in the population and was also found to multiply faster and to induce a more severe inflammation process in the lungs of gnotobiotic pigs. Further studies will be necessary to identify specific virulence factors of the organism responsible for these findings. It was also concluded that vaccination is not able to significantly reduce the transmission of *M. hyopneumoniae*. The currently used vaccines will therefore not result in eradication of the organism. Therefore, other measures will also be necessary and/or new vaccines which are able to reduce the colonization of the respiratory tract more significantly are needed to achieve eradication. The transmission model developed here can be used to evaluate the impact of such control measures on the transmission of the disease.
SAMENVATTING

*Mycoplasma hyopneumoniae (M. hyopneumoniae)* is het primaire agens voor Enzoötische Pneumonie bij varkens. Het speelt een sleutelrol in het Porcien Respiratoir Ziektecomplex bij varkens. Infecties met *M. hyopneumoniae* leiden tot chronische ademhalingsaandoeningen die wereldwijd aanwezig zijn. Enkelvoudige infecties zijn relatief goedaardig, maar onder praktijkomstandigheden fungeert de kiem als een gangmaker voor gecompliceerde ademhalingsaandoeningen. Deze zijn verantwoordelijk voor belangrijke economische schade in de varkenshouderij. Door de wereldwijde prevalentie en de hoge en langdurige financiële verliezen wordt *M. hyopneumoniae* aanzien als één van de belangrijkste ziekteverwekkers in de varkenshouderij.


De epidemiologie met betrekking tot het transmissiepatroon van *M. hyopneumoniae* is duidelijk beschreven, maar de transmissie van de kiem was nog niet gekwantificeerd onder experimentele omstandigheden. Kennis omtrent de spreiding van *M. hyopneumoniae* bij jonge biggen en inzicht in de factoren die deze verspreiding beïnvloeden, zijn noodzakelijk om de controle van de infectie te optimaliseren.
Recent werden hoog- en laagvirulente isolaten van *M. hyopneumoniae* beschreven. Tot nog toe is er geen informatie beschikbaar omtrent mogelijke verschillen in de interactie van deze *M. hyopneumoniae* isolaten met het ademhalingsstelsel van varkens.

Het doel van de hier voorliggende thesis was een beter inzicht te verwerven in de transmissie van *M. hyopneumoniae* tijdens de opfok van de biggen op de batterij. Hiervoor werd een experimenteel transmissiemodel ontwikkeld. Het verschil in overdracht tussen hoog- en laagvirulente isolaten werd onderzocht. Het model werd ook gebruikt om het effect van vaccinatie op de transmissie te evalueren. Daarenboven werden de kiem-gastheer interacties van het hoog- en laagvirulent isolaat onderzocht, samen met mogelijke mechanismen verantwoordelijk voor het verschil in virulentie.

In de eerste studie (Hoofdstuk 2.1.1.), werd de transmissie van twee *M. hyopneumoniae* isolaten onderzocht en gekwantificeerd onder experimentele omstandigheden. Een aangepaste reproductieratio werd berekend (Rn). Deze waarde, berekend volgens de ‘final size’ methode, geeft het gemiddeld aantal secundaire infecties weer ten gevolge van één typisch infectieuze big tijdens de opfokperiode. Deze periode, van 4 tot 10 weken leeftijd, komt overeen met de conventionele batterijperiode in West-Europese productiesystemen. Daarnaast werd de spreiding van hoog- en laagvirulente isolaten vergeleken.

Achtenveertig pas gespeende biggen, vrij van *M. hyopneumoniae*, werden in 6 verschillende afdelingen gehuisvest. Gedurende 6 weken werden 2 experimenteel geïnoculeerde dieren bij 6 gevoelige biggen geplaatst. Op het einde van de observatieperiode werd het aantal contact-geïnfecteerde biggen bepaald op basis van nPCR positieve longspoelsels. De Rn-waarden van het hoog- en laagvirulent isolaat waren respectievelijk 1,47 (0,68 - 5,38) en 0,85 (0,33 - 3,39). Er werd geen significant verschil gevonden tussen beide groepen (P = 0,53). De algemene Rn was 1,16 (0,94 - 4,08). De conclusie, onder de door ons toegepaste experimentele omstandigheden, was dat een big, geïnfecteerd vóór het spenen, minstens één toomgenoot zal besmetten tijdens de batterijperiode.
Zoals hoger vermeld, wordt de ziekte onder controle gehouden door vaccinatie, maar de invloed van vaccinatie op de spreiding van de kiem zelf werd nog niet geëvalueerd. In hoofdstuk 2.1.2. werd een transmissie experiment uitgevoerd om het effect van vaccinatie op de transmissie te evalueren door middel van een aangepaste reproductieratio. Dertig biggen, gevaccineerd op de leeftijd van 1 week en 30 niet-gevaccineerde biggen, vrij van *M. hyopneumoniae*, werden gehuisvest in 6 afzonderlijke afdelingen. In elke afdeling werden 3 biggen eerst intratracheaal geïnoculeerd met *M. hyopneumoniae* en daarna samengeplaatst met 7 gevoelige contactdieren. De transmissie werd geëvalueerd gedurende de conventionele batterijperiode. Op het einde van de studie werd de infectiestatus van de dieren bepaald op basis van de resultaten van nPCR onderzoek op longspoelvocht. De R₉₅-waarde in de gevaccineerde groep was 2,38 (1,07 - 7,53), terwijl in de niet-gevaccineerde groep een R₉₅-waarde van 3,51 (1,51 - 9,34) werd waargenomen. Beide waarden waren niet significant verschillend van elkaar (*P* = 0,77). Onder de door ons toegepaste experimentele omstandigheden was de transmissie van *M. hyopneumoniae* slechts licht gereduceerd in de gevaccineerde groepen. Vaccinatie kon daarenboven niet verhinderen dat *M. hyopneumoniae* koloniseert ter hoogte van de longen.

In hoofdstuk 2.2 werd de interactie onderzocht van een hoog- en een laagvirulent isolaat met de longen van biggen. Om beter inzicht te verwerven in de mechanismen verantwoordelijk voor het waargenomen verschil in virulentie tussen beide *M. hyopneumoniae* isolaten, werden 23 biggen, geboren via een keizersnede en colostrumvrij opgegroeid, onderverdeeld in 3 groepen. Groepen 1 en 2 bestonden uit elk 9 biggen die intratracheaal geïnoculeerd werden op de leeftijd van 1 week met respectievelijk een hoog- of een laagvirulent isolaat. De overige 5 biggen werden geïnoculeerd met steriel cultuurmedium. Dieren werden geëuthanaseerd op 5, 10, 15 en 28 dagen na de experimentele inoculatie (DPI). Dieren geïnoculeerd met het hoogvirulent isolaat hadden meer neutrofielen in hun longspoelsel op 10, 15 en 28 DPI, in vergelijking met de andere groepen. Op 10 en 15 DPI
hadden de dieren in de hoogvirulente groep significant hogere TNF-α concentraties in het longspoelsel. De IL-1 concentratie was in dezelfde groep hoger op 5 en 28 DPI in vergelijking met de andere groepen. Vanaf 10 DPI werden significant hogere titers van *M. hyopneumoniae* waargenomen in longspoelsels van dieren geïnoculeerd met het hoogvirulent isolaat, in vergelijking met dieren geïnoculeerd met het laagvirulent isolaat. Met deze studie werd aangetoond dat het verschil in virulentie tussen de hoog- en de laagvirulente isolaten geassocieerd was met een snellere *in vitro* groei van *M. hyopneumoniae*, een hogere vermeerderingscapaciteit ter hoogte van de longen en een snellere inductie van een erger ontstekingsproces door het hoogvirulent isolaat.

Uit deze studies kan geconcludeerd worden dat *M. hyopneumoniae* zich relatief traag verspreidt tijdens de batterijperiode. Een hoogvirulent isolaat heeft een licht, maar niet significant, hogere capaciteit om te spreiden in de populatie, vertoont ook een snellere vermeerdering in de longen van kiemvrije biggen en induceert een ergere ontstekingsreactie dan het laagvirulent isolaat. Er werd ook vastgesteld dat vaccinatie niet zal leiden tot een sterke reductie van de spreiding van *M. hyopneumoniae*. Met de huidige vaccins kan geen eradicatie van de kiem plaatsvinden. Wanneer men *M. hyopneumoniae* wil eradiceren, zullen ook andere maatregelen nodig zijn en/of zullen er nieuwe vaccins ontwikkeld moeten worden die de kolonisatie van *M. hyopneumoniae* ter hoogte van de longen kunnen verhinderen. Het ontwikkelde transmissiemodel kan gebruikt worden om de impact van controlemaatregelen op de spreiding van de ziekte te onderzoeken. Verder onderzoek zal nodig zijn om de specifieke virulentiefactoren te onderscheiden die gerelateerd zijn met het induceren van de letsels. De identificatie van deze factoren kan gebruikt worden voor de ontwikkeling van nieuwe en betere vaccins.
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Tom.
CURRICULUM VITAE
PERSONALIA


In 2006 behaalde hij het post-graduaatdiploma van ‘Vakdierenarts Varken’ met grote onderscheiding en kreeg hij voor zijn scriptie de Boehringer Ingelheim-prijs voor Vakdierenarts Varken.

Tom Meyns is auteur of medeauteur van meerdere publicaties in nationale en internationale tijdschriften. Hij was ook spreker op verschillende nationale en internationale congressen.
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