

Treatment and control of *Mycoplasma hyopneumoniae* infections

Rubén del Pozo Sacristán

A mis padres

Everyone is a genius.

But if you judge a fish on its ability to climb a tree,
it will live its whole life believing that it is stupid.

Albert Einstein

Todo el mundo es un genio.

Pero si juzgas a un pez por su habilidad de escalar un árbol,
pasará toda su vida pensando que es estúpido.

Albert Einstein



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LIST OF ABBREVIATIONS

ADG	Average daily weight gain
ANOVA	Analysis of variance
BAL	Broncho-alveolar lavage
CCU	Color changing units
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FCR	Feed conversion ration
ID	Intradermal
IF	Immunofluorescence
Ig	Immunoglobulins
IM	Intramuscular
MIC	Minimum inhibitory concentration
nPCR	nested Polymerase chain reaction
NV	Non-vaccinated
NS	Nasal swabs
OD	Optical density
PCR	Polymerase chain reaction
PCV-2	Porcine circovirus type 2
ppm	parts per million
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
RDS	Respiratory disease score
qPCR	quantitative Polymerase chain reaction
SD	Standard deviation
SIV	Swine influenza virus
TBS	Tracheo-bronchial swabs
U.K.	United Kingdom
U.S.	United States
V	Vaccinated

Chapter 1. **GENERAL INTRODUCTION**

General introduction

REVIEW OF THE LITERATURE

Respiratory disease in pigs is one of the most important disease conditions in intensive pig production systems worldwide. *Mycoplasma hyopneumoniae* is the primary agent of enzootic pneumonia, a chronic respiratory disease in pigs resulting from mixed respiratory infections with *M. hyopneumoniae* and one or more secondary bacterial pathogens (Maes *et al.*, 2008a). *M. hyopneumoniae* is present in most of the countries with intensive swine production and is also considered as one of the main pathogens involved in the porcine respiratory disease complex (PRDC). The multifactorial aetiology of the PRDC includes both viral and bacterial pathogens, and is also influenced by management and environmental conditions (Opriessnig *et al.*, 2011). Both EP and PRDC lead to major economic losses due to the reduced growth, increased mortality and feed conversion, costs for antimicrobials and immunoprophylaxis and increased time to market (Maes *et al.*, 2008a).

M. hyopneumoniae lacks a cell wall and has a small genome encoding only a few biosynthetic pathways. The pathogenesis of *M. hyopneumoniae* infections includes various steps: adhesion of the respiratory tract, modulation of the immune system leading to damage of pulmonary tissue and persistence in the respiratory tract, as well as interaction with other infectious agents.

Non-productive coughing is the most obvious clinical sign. Macroscopic lesions are characterized by catarrhal bronchopneumonia. They consist of red to purplish consolidated areas on the cranial-ventral parts of the apical, cardiac, accessory and diaphragmatic lobes. The diagnosis of enzootic pneumonia is generally made at herd level rather than at individual level (Thacker and Minion, 2012; Taylor, 2013).

Since eradication of *M. hyopneumoniae* from infected herds is complicated and difficult to achieve, control of the infections is considered the best strategy. Control measures include optimization of housing and management practices, antimicrobial treatment and vaccination. This review aims to summarize the current knowledge on *M. hyopneumoniae* infections and enzootic pneumonia, with emphasis on antimicrobial agents used for treatment and vaccines used for control this disease.

1.1. CHARACTERISTICS OF *MYCOPLASMA HYOPNEUMONIAE*

Mycoplasmas are taxonomically classified as members of the class Mollicutes, a group of bacteria characterized by the lack of a cell wall. Mycoplasma cells are mostly spherical and range from 0.3 to 0.8 μm in size (Razin, 2006). They have a small genome (893-920 kilobase pairs) (Dybvig and Voelker, 1996), which encodes a limited number of genes, resulting in a few biosynthetic pathways (Razin *et al.*, 1998). Because of this limited metabolism, mycoplasmas need to obtain essential metabolites from the host or growth environment (Thacker and Minion, 2012).

The first isolation of *M. hyopneumoniae* was performed in 1965 in the U.S. (Mare and Switzer, 1965) and the U.K. (Goodwin *et al.*, 1965). In the 70's of the previous century, important research was carried out to determine the main characteristics of this organism. *M. hyopneumoniae* is very sensitive to environmental conditions due to the absence of a cellular wall (Goodwin, 1972). In 1973, the J-strain was isolated (Whittlestone, 1973), which is still considered as the reference strain. Due to its slow and fastidious growth *in vitro* (Friis, 1974), a special culture medium supplemented with serum, Friis medium, is necessary for isolation (Friis, 1975). Using electron microscopy, *M. hyopneumoniae* appears as round or oval cells with a mean diameter ranging from 0.5 to 0.8 μm (Tajima and Yagihashi, 1982; Blanchard *et al.*, 1992).

The whole genome sequence of several *M. hyopneumoniae* strains has been published (strains J, 232, 7448 and 168) (Liu *et al.*, 2011; Minion *et al.*, 2004; Vasconcelos *et al.*, 2005). Recent studies using molecular techniques have revealed a large diversity of *M. hyopneumoniae* isolates at genomic (Stakenborg *et al.*, 2006b; Strait *et al.*, 2008; Nathues *et al.*, 2011), and proteomic (Vicca *et al.*, 2003; Calus *et al.*, 2007; Pinto *et al.*, 2007) level. Different *M. hyopneumoniae* isolates also have different virulence characteristics, as assessed by experimental infections in pigs (Vicca *et al.*, 2003; Villarreal *et al.*, 2009).

1.2. PATHOGENESIS OF *MYCOPLASMA HYOPNEUMONIAE* INFECTIONS

M. hyopneumoniae is a host specific organism that only infects pigs. The pathogenesis of *M. hyopneumoniae* infections includes various steps: colonization of the respiratory tract, modulation of the immune system leading to damage of pulmonary tissue and persistence in the respiratory tract. This complex pathogenesis may also be affected, mainly under field conditions, by the interaction with other infectious agents.

Colonization of the respiratory tract begins with the adherence of *M. hyopneumoniae* to the ciliated epithelial cells of the trachea, bronchi and bronchioles (Blanchard *et al.*, 1992). This attachment to the mucosal surface of the ciliated epithelium is a pre-requisite for initiation of disease (Tajima and Yagihashi, 1982) and is mainly enabled by various proteins (*e.g.* P97, P102, P116, P135, P159, P216) and other cell surface structures. A complete description of these multifunctional adhesins and their binding abilities can be found in Simionatto *et al.* (2013). The binding of *M. hyopneumoniae* to the cilia provokes ciliostasis, clumping and finally loss of cilia (DeBey and Ross, 1994). The impaired ciliary activity leads to a reduction of the mucosal clearance of the respiratory tract. Once the first non-specific defense barrier of the lung is damaged, further multiplication of the *M. hyopneumoniae* and colonization of the airways occur. The loss of the cilia is predominantly limited to the airways from cranio-ventral lobes of the lungs (apical and cardiac) and this distribution is associated also with the main location of macroscopic lesions of pneumonia (Mebus and Underdahl, 1977).

It has been shown that *M. hyopneumoniae* is able to **modulate both innate and adaptive immune responses** (Thacker, 2001), leading to evasion of host defenses and establishment of a chronic and persistent infection (Razin *et al.*, 1998). The exact mechanism of modulation of the immune response is not yet fully elucidated (Simecka, 2005), but it is generally accepted that the immune response plays a role in the development of lesions. Immediately after colonization, there is a massive infiltration of peribronchiolar and perivascular lymphohistiocytic cells (Morris *et al.*, 1995). Alveolar macrophages and lymphocytes are the predominant cells. *M. hyopneumoniae* has the ability to alter the function of these cells. The phagocytic capacity of the macrophages is impaired, resulting in a reduced clearance, and consequently, in a chronic colonization of the respiratory tract by *M. hyopneumoniae* (Caruso and Ross, 1990). In addition, *M. hyopneumoniae* stimulates macrophages to produce proinflammatory cytokines, which leads to an inflammatory

response and lung damage (Thacker and Minion, 2012). A general immunosuppression has been suggested since *M. hyopneumoniae* may also alter the function of lymphocytes (Kishima and Ross, 1985).

M. hyopneumoniae usually persists for a long time in the animal. Sorensen *et al.* (1997) demonstrated the presence of *M. hyopneumoniae* in the respiratory tract by means of Polymerase Chain Reaction (PCR) up to 85 days post-infection. Using nested PCR (nPCR), Fano *et al.* (2005) detected *M. hyopneumoniae* in nasal swabs up to 185 days post-infection. More recently, Pieters *et al.* (2009) showed that *M. hyopneumoniae* can be isolated from the respiratory tract of potential infectious carriers up to 200 days post-infection, it can persist up to 214 days post-infection, and total clearance is only complete by 254 days post-infection.

The complete pathogenesis of *M. hyopneumoniae* infections is not yet fully understood, since not all respiratory tract infections with *M. hyopneumoniae* lead to pneumonia. Other factors, such as virulence of the strain involved (Zielinski and Ross, 1990; Vicca *et al.*, 2003; Meyns *et al.*, 2007; Villarreal *et al.*, 2009) and interactions with other respiratory pathogens (Ciprián *et al.*, 1994; Opriessnig *et al.*, 2011), may play a decisive role in the development of the disease.

1.3. EPIDEMIOLOGY OF *MYCOPLASMA HYOPNEUMONIAE*

1.3.1. Occurrence of *M. hyopneumoniae* infections

M. hyopneumoniae is present in almost all countries with an intensive swine production (Sibila *et al.*, 2004a; Fano *et al.*, 2007; Maes *et al.*, 2008a; Martínez *et al.*, 2009; Fraile *et al.*, 2010; Meyns *et al.*, 2011; Fablet *et al.*, 2012b; Nathues *et al.*, 2012b), and within herds, infections may occur in all the production phases, namely breeding animals, suckling and weaned piglets, as well as fattening pigs.

The prevalence of *M. hyopneumoniae* in **suckling pigs** assessed by nPCR on nasal swabs ranges from 0.5 to 10 per cent (Sibila *et al.*, 2007a; Sibila *et al.*, 2007b; Villarreal *et al.*, 2010). The prevalence is higher in **weaned piglets**, namely from 0 to 51 per cent (Sibila *et al.*, 2004a; Fano *et al.*, 2007; Sibila *et al.*, 2007b). Even though infections may already occur during suckling period, in most pig herds, the highest infection levels of *M. hyopneumoniae* occur during the **grow-finishing period** (Sibila *et al.*, 2004a). The dynamics of infection vary largely between herds, and are influenced by different environmental conditions, such as management and housing conditions and the production system of the herd. The potential of the sows to shed *M. hyopneumoniae* is one of the key points on the epidemiology of this agent, not only because of the transmission to their offspring (Calsamiglia and Pijoan, 2000), but also in the maintaining of the infection within the herd. There is however not much information available on occurrence of *M. hyopneumoniae* in **breeding animals** (Sibila *et al.*, 2009). The prevalence of *M. hyopneumoniae* in sows assessed by antibodies in sera ranges from 27 to 65 per cent (Sibila *et al.*, 2007a; Grosse Beilage *et al.*, 2009).

1.3.2. Transmission of *M. hyopneumoniae*

The transmission of *M. hyopneumoniae* can take place by different routes. Transmission between herds occurs mainly by the introduction of infected animals or airborne. Regarding the transmission within herds, distinction can be made between vertical (from the sow to the offspring) and horizontal transmission (between pigs of the same or different pens).

Vertical transmission

Vertical transmission occurs predominantly via nose-to-nose contact (Calsamiglia and Pijoan, 2000). To date, in-utero or lactogenic transmission has not been reported (Maes *et al.*,

2008b). Moore *et al.* (2001) suggested a reduction of the severity of pneumonia lesions in slaughter pigs born from parity 2 sows when compared with pigs born from parity 1 sows. Recent epidemiological studies confirmed these observations and have demonstrated that the piglets from younger sows (gilts and parity 1-2) have more risk of being infected with *M. hyopneumoniae* compared to piglets from older sows (Fano *et al.*, 2007). Although the risk is lower than in younger sows, older sows may still transmit *M. hyopneumoniae* to their offspring (Calsamiglia and Pijoan, 2000; Grosse Beilage *et al.*, 2009).

Horizontal transmission

a. Direct contact:

Nose-to-nose contact between penmates or even between pigs of different pens may result in spread of *M. hyopneumoniae* from infected to susceptible animals (Thacker and Minion, 2012). Using transmission experiments under experimental conditions, Meyns *et al.*, (2004) showed that one infected pig may be able to infect one littermate during a nursery period of six weeks. Similar results were obtained by Villarreal *et al.*, (2011) in transmission experiments under field conditions.

b. Indirect contact:

Airborne transmission has been shown to be important for *M. hyopneumoniae*. Goodwin (1985) stated that *M. hyopneumoniae*-free herds may become infected with *M. hyopneumoniae* if infected herds are located within a distance of 3.2 km. Cardona *et al.* (2005) showed under experimental conditions using aerosols that transmission of *M. hyopneumoniae* is possible over 150 m. More recent studies have demonstrated that *M. hyopneumoniae* may be transmitted over much larger distances namely 4.7 km (Dee *et al.*, 2009) and 9.2 km (Otake *et al.*, 2010).

The implementation of standard hygiene measures of the personnel when entering the herd may reduce the risk of transmission of *M. hyopneumoniae* between herds (Batista *et al.*, 2004). More recently, an observational study performed during four years illustrated that one-night downtime period prevents the entry of *M. hyopneumoniae* in a herd not only by personnel, but also by fomites (Pitkin *et al.*, 2011). In general, mechanical vectors, such fomites and personnel, are considered to be of limited importance in the transmission of *M. hyopneumoniae*.

1.4. CLINICAL SIGNS, LESIONS AND PORCINE RESPIRATORY DISEASE COMPLEX

1.4.1. Clinical signs

In general, two distinct forms of clinical disease can be observed, namely endemic and epidemic disease (Thacker and Minion, 2012; Taylor, 2013).

Endemic mycoplasmosis, commonly known as **enzootic pneumonia**, is the form most frequently observed. In many cases, infections are subclinical (Clark *et al.*, 1991). In case of clinical disease, a non-productive coughing is the most obvious clinical sign. Coughing may be present in nursery, grower and finisher pigs and usually a considerable percentage of the pigs are affected. Coughing may disappear after 2-3 weeks, but it can also persist throughout the whole fattening period.

Under experimental infections, clinical signs are mainly characterized by slight fever, followed by dry coughing. Coughing appears from 10 to 14 days post-infection, reaches a maximum peak at about 4-5 weeks, after which it disappears gradually (Whittlestone 1972; Kobisch *et al.*, 1993).

Under field conditions, herds that are only infected with *M. hyopneumoniae* are rarely seen. Mostly, mixed infections occur. In case of secondary bacterial infections, the severity of the clinical signs increases, namely a severe respiratory distress including fever, labored breathing and prostration, and reduced appetite. This leads to an increase of the feed conversion ratio, lower average daily weight gain, and more weight variation between the pigs.

Epizootic mycoplasmosis is uncommon. It occasionally occurs when *M. hyopneumoniae* enters into a negative, immunologically naive herd. The spread of the disease occurs rapidly and all age groups may be affected. Coughing, acute respiratory distress, fever and deaths may be present (Thacker and Minion, 2012).

1.4.2. Lesions

M. hyopneumoniae infection can lead to different gross lesions depending on the course of the disease. The most common macroscopic lesion in chronic *M. hyopneumoniae* infections is characterized by red to purplish consolidated areas on the cranial-ventral parts of the apical, cardiac, accessory and diaphragmatic lobes. These lesions are commonly called *Mycoplasma*-like pneumonia lesions (active lesions) (Figure 1) (Whittlestone, 1972; Kobisch *et al.*, 1993). They may appear from 7 days after experimental infection onwards and reach a

maximum size at about 4 weeks post-infection (Whittlestone, 1972; Kobisch *et al.*, 1993). These uncomplicated lesions are characterized by the presence of a catarrhal exudate in the airways, a uniform colour of the parenchyma of the lung and a “meaty” consistency. The pneumonia lesions may be recovered after 7-10 weeks, and by that time, red to purplish interlobular scar retractions of connective tissue, called fissures, are present (old lesions) (Whittlestone, 1972; Kobisch *et al.*, 1993).

Under field conditions, when *M. hyopneumoniae* infections are complicated by secondary bacterial pathogens, the pneumonia lesions occupy a large surface of the lung, and are characterized by the presence of mucopurulent exudate in the airways, a more firm consistency and an inconsistent greyish colour of the parenchyma (Osborne *et al.*, 1981). However, *Mycoplasma*-like pneumonia lesions at slaughter are not pathognomonic for infections with *M. hyopneumoniae*. Apart from *M. hyopneumoniae*, it is well known that infections *e.g.* with viruses and in particular swine influenza virus, may cause similar pneumonia lesions (Thacker *et al.*, 2001, Sibila *et al.*, 2009).

Adhesive pleurisy is a common finding in slaughter pigs reared under field conditions and has been associated with respiratory disease (Fraile *et al.*, 2010; Meyns *et al.*, 2011; Fablet *et al.*, 2012b; Merialdi *et al.*, 2012). It is defined as fibrotic adherence between the visceral and parietal membranes of the pleural sac. Although adhesive pleurisy is not a characteristic lesion of *M. hyopneumoniae* infections, Meyns *et al.* (2011) found a positive association between higher prevalence of pneumonia and the presence of pleurisy at slaughter.

Histopathological examination of the pneumonia lesions infected with *M. hyopneumoniae* revealed neutrophil and lymphocyte infiltration around the airways and alveoli (Whittlestone, 1972). As the infection progresses, broncho-interstitial pneumonia develops. The accumulation of mononuclear cells, mainly lymphocytes and macrophages, is more evident and results in the formation of lymphocytic cuffs around the airways, which is commonly known as perivascular and peribronchiolar lymphohistiocytic infiltration and nodule formation (Blanchard *et al.*, 1992; Morris *et al.*, 1995). In cases of enzootic pneumonia, a neutrophilic exudate may be present in the lumen of airways and alveoli.

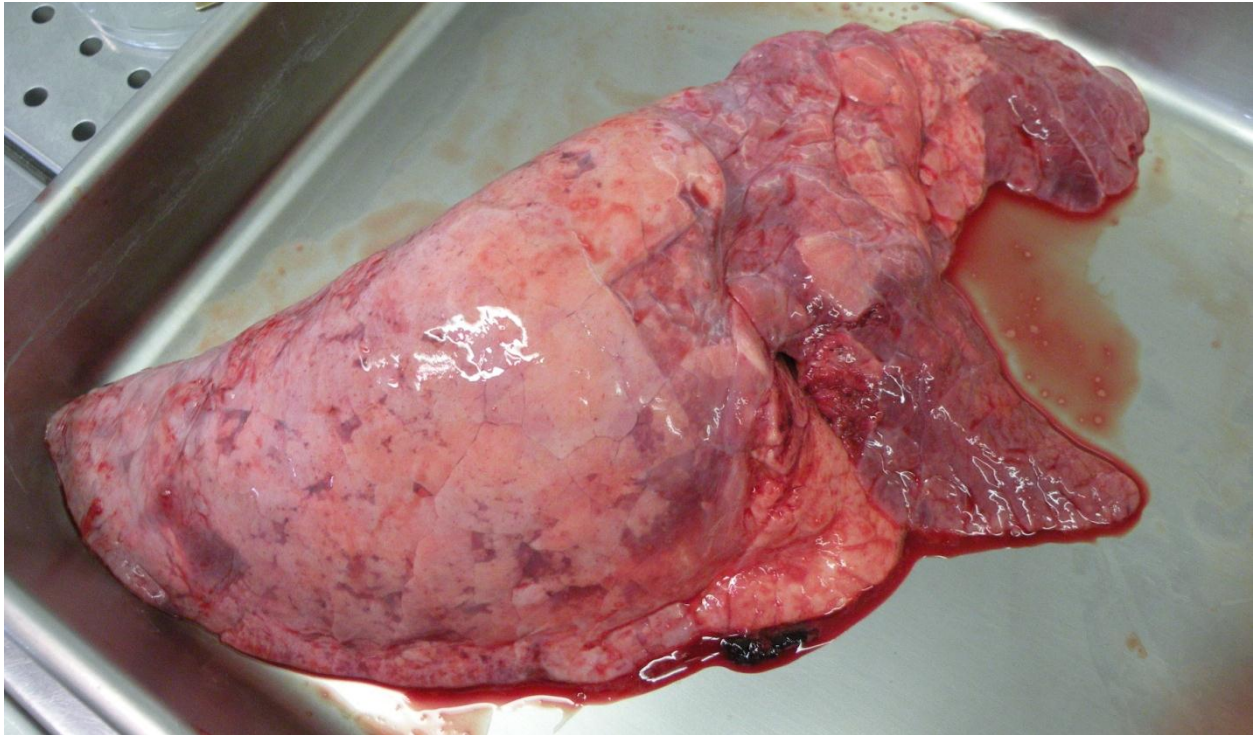


Figure 1. Red to purplish consolidated areas on the cranial-ventral parts of the apical, cardiac, accessory and diaphragmatic lobes, called *Mycoplasma*-like pneumonia lesions.

1.4.3. Porcine respiratory disease complex

During the past 40 years, swine production has experienced an important intensification, mainly based on larger pig herds operating under conditions of confinement, which leads to an increase of respiratory disease. Respiratory disease in swine is most often due to the interaction and synergy of primary and opportunistic infectious agents, both viral and bacterial pathogens. The severity of this respiratory disease may be exacerbated when adverse environmental and management conditions are present. This multifactorial nature of respiratory disease in pigs is called the Porcine Respiratory Disease Complex (PRDC) (Brockmeier *et al.*, 2002).

The term PRDC was coined during the 90's in U.S. to describe pneumonia of multiple etiology causing clinical respiratory disease and poor performance later in the finishing period (15 to 20 weeks) (Halbur and Andrews, 1993; Dee, 1996). Shortly afterwards, it was observed that it most often occurred 8 to 10 weeks following placement into the finishing facilities and PRDC was commonly referred to as the "18 to 20 week wall" (Dee, 1997). Nowadays, this term may also refer to pneumonia of mixed etiology at other production stages, such as in weaned, nursery or grower pigs, and not only during the finishing period. However, in these cases the correct terminology may be mixed respiratory disease. Currently,

it is generally accepted that when *M. hyopneumoniae* is the primary agent of respiratory disease and other secondary bacteria take advantage of this situation, the resulting disease is called enzootic pneumonia. On the other hand, in case of PRDC, *M. hyopneumoniae* plays a central role together with viral pathogens such as Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Porcine Circovirus type 2 (PCV-2) and Swine Influenza Virus (SIV), whereas other viruses and opportunistic bacteria are considered as secondary actors (Opriessnig *et al.*, 2011).

1.4.3.1. Polymicrobial nature

Numerous studies have illustrated during the past years the polymicrobial nature of PRDC (Morrison *et al.*, 1985a; Loeffen *et al.*, 1999; Bochev, 2007; Moorkamp *et al.*, 2008; Hansen *et al.*, 2010; Opriessnig *et al.*, 2011; Fablet *et al.*, 2012a; Ticó *et al.*, 2013). There is an enormous variety of microorganisms associated with PRDC and they can be categorized as commensal microflora vs potentially pathogenic agents; primary vs secondary or opportunistic pathogens. There is no clear distinction between commensal and potential pathogenic agents, since different studies categorized the same agent as either commensal or pathogenic (VanAlstine, 2012). Primary pathogens are capable of evading respiratory defense mechanisms from the host and establishing infection and disease on their own, whereas secondary pathogens take advantages of the damage caused by primary pathogens to establish infections and exacerbate respiratory disease (Brockmeier *et al.*, 2002). In general, primary pathogens, such as viral agents and *M. hyopneumoniae*, may damage the epithelium of the respiratory tract and decrease the local and systemic defense mechanisms, favoring invasion, colonization and establishment of the infection by secondary agents, mainly bacteria. Primary pathogens involved in PRDC include PRRSV, PCV-2, SIV, Aujeszky's Disease Virus, *M. hyopneumoniae*, *Actinobacillus pleuropneumoniae* and *Bordetella bronchiseptica*. Other pathogens include Paramyxovirus, Porcine Cytomegalovirus, Porcine Respiratory Coronavirus, Torque teno sus virus, *Actinobacillus suis*, *Haemophilus parasuis*, *M. hyorhinis*, *Pasteurella multocida*, *Streptococcus suis* and *Trueperella pyogenes* (Opriessnig *et al.*, 2011). Major efforts have been done to study and understand the interaction between different respiratory pathogens under experimental conditions. Although the reproduction of disease has been easily achieved by the use of single challenge infection models, it is not always possible to reproduce disease in dual infections or co-infection experiments. A review was published by Opriessnig *et al.* (2011) where a wide variety of interactions between different viral and bacterial pathogens is discussed.

Under field conditions, it is very rare to observe a clinical case of respiratory disease in which only one primary pathogen is involved. Frequently, one or more primary agents are present and cause primary infections which become complicated with opportunistic bacteria that aggravate the respiratory disease (VanAlstine, 2012). A study published in the U.S. including three case reports of PRDC (Harms *et al.*, 2002) illustrated a low morbidity and mortality. Postmortem examinations revealed a high extent of pneumonia lesions and bronchopneumonia as major gross and microscopic findings, respectively. Concurrent infections of PCV-2 combined with PRRSV, SIV and *M. hyopneumoniae* were described.

1.4.3.2. Non-infectious factors

Apart from its polymicrobial nature, PRDC also depends largely on many non-infectious factors. Identifying and reducing risk factors may lead to a reduction of the transmission of pathogens, less stress provoked to the animal by hostile environments (physical, climate and air quality factors) and less direct impairment of the respiratory tract (Opriessnig *et al.*, 2011). A short description of non-infectious factors is presented in section 1.6 of this thesis. Details on the influence of management and environmental factors on respiratory disease are also well described in the review papers by Stärk (2000) and Maes *et al.* (2008a).

1.5. DIAGNOSIS

Diagnosis of enzootic pneumonia is generally established at group level rather than at individual level (Thacker and Minion, 2012; Taylor, 2013). The presence of coughing in fattening pigs combined with chronic bronchopneumonia lesions at slaughter, as well as poor performance and feed efficiency during the fattening period may lead to a presumptive diagnosis of enzootic pneumonia. However, definitive diagnosis is based on detection of (parts of) *M. hyopneumoniae*. Differential diagnosis should consider other possible causes of coughing, as well as bacteria and viruses causing *Mycoplasma*-like pneumonia lesions (Sibila *et al.*, 2009).

1.5.1. Clinical signs

Non-productive coughing, especially in fattening pigs, being more evident after boosting and characterized by persistence in the population is a typical sign of enzootic pneumonia.

Several methods have been described to assess the severity of coughing under experimental and field conditions. Halbur *et al.* (1996) described a Respiratory Disease Score (RDS) to quantify the severity of coughing under experimental conditions. The RDS could range from 0 to 6: 0 (no coughing), 1 (mild coughing after encouraged move), 2 (mild coughing in rest), 3 (moderate coughing after encouraged move), 4 (moderate coughing at rest), 5 (severe coughing after encouraged move), 6 (severe coughing at rest). This method has been used in recent transmission and pathogenesis studies, as well as in the assessment of efficacy of vaccines and antimicrobial treatments (Vicca *et al.*, 2003; Meyns *et al.*, 2004; Vicca *et al.*, 2005; Meyns *et al.*, 2006; Meyns *et al.*, 2007). Under field conditions, a method that quantifies the number of pigs coughing in a given period of time has also been described (Maes *et al.*, 1999; Mateusen *et al.*, 2001). The pigs were moved up and the number of pigs coughing per pen during 10 minutes was counted. A RDS was calculated by dividing the number of pigs per pen that coughed during 10 min, by the total number of pigs in that pen, multiplied by 100. A similar method has been used by Nathues *et al.* (2012a). These authors confirmed that a quantitative assessment of the onset of coughing is useful for the diagnosis of enzootic pneumonia and is correlated with the infection pattern of *M. hyopneumoniae* assessed by PCR.

1.5.2. Macroscopic lung lesions

Examination of the lungs of slaughter pigs is an excellent method to monitor the respiratory health in a herd under field conditions. If possible, checks should include visual examination and palpation in a well-illuminated place, and the lesions should be sketched onto a diagram, which may be followed by image analysis (VanAlstine, 2012). The prevalence and extent of the lesions, not only pneumonia, but also adhesive pleurisy, nodules, abscesses, pericarditis, etc., may provide reliable information regarding the severity of the respiratory disease.

However, slaughterhouse examinations also have limitations. Lesion assessment is subjective and the lesions are generally not pathognomic (Sibila *et al.*, 2009). Subclinical infections may be not detected and lesions in young animals may be healed at the moment of slaughter (Regula *et al.*, 2000). Also, the fast speed of slaughtering in modern slaughter facilities makes it more difficult to perform reliable slaughter checks. Although a minimum of 30 randomly selected pigs from the same slaughter batch has been shown to provide reliable information at herd level (Straw *et al.*, 1989), it is generally accepted that it is better to evaluate more pigs. In recent epidemiological studies performed in Europe, in which slaughter examinations were carried out in order to estimate the prevalence and severity of lung lesions, an average of 127 (Meyns *et al.*, 2011), 106 (Fraile *et al.*, 2010), 100 (Merialdi *et al.*, 2012) and 30 (Fablet *et al.*, 2012) pigs per batch were investigated. The low number of the last study may be due to the large amount of additional bacteriological investigations carried out on these lungs.

Different scoring systems have been described in the past years to monitor enzootic pneumonia under experimental (Hanan *et al.*, 1982), as well as field conditions (Madec *et al.*, 1982; Morrison *et al.*, 1985b; Straw *et al.*, 1986; Christensen *et al.*, 1999). Only the methods used in this thesis will be described here. The scoring system described by Hannan *et al.* (1982) is appropriate to carefully quantify lung lesions caused by experimental infection with *M. hyopneumoniae*. The score ranges from 0 (no lesions) to 35 (all lung tissue affected). This method is especially designed for *Mycoplasma*-like pneumonia lesions, since they are mainly located in the cranial-ventral parts of the lungs, and this score gives the same significance to each of the 7 lobes, namely 5 points/lobe out of the total are of the lung (35 points). The lung lesion score from Morrison *et al.* (1985b) is appropriate for field evaluation. It is based on the total percentage of affected lung tissue, with the different lung lobes representing the

following percentage of the total lung surface area: apical (10 per cent), cardiac (7 per cent), accessory (6 per cent) and diaphragmatic (30 per cent).

1.5.3. Microscopic lung lesions

Microscopic findings of peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation are not specific of *M. hyopneumoniae* infections, but they may be helpful for a final diagnosis. Two methods can be performed to measure the severity and the extent of the mycoplasmal lesions.

The severity of peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation related to *M. hyopneumoniae* induced pneumonia lesions may be scored by using light microscopy according to the method described by Morris *et al.* (1995). The score ranges from 1 (limited cellular infiltrates around bronchioles) to 5 (severe infiltration of lymphocytic cells around bronchioles and interstitium, and inflammatory cell exudates into the airways) (Fig. 2).

The extent of pneumonia can also be measured by calculating the percentage of tissue occupied by air. Using a camera software and light microscopy, a picture is taken from a microscopic field including a sample of lung lesion. By means of an automatic image analysis system, the percentage of air in each picture is calculated. This percentage is negatively correlated to the number of infiltrating cells, the amount of atelectasis, intrabronchiolar-intrabronchial exudates, intra-alveolar exudates and/or oedema and proliferation of type II cells. The advantage of this method is that the measurements are done in an objective way.

1.5.4. Demonstration of *M. hyopneumoniae* infections

Bacteriological culture from fresh lung tissue is considered as the gold standard for diagnosis of enzootic pneumonia. However, due to the fastidious growth of *M. hyopneumoniae*, the very time-consuming isolation procedure, the fact that special media are required and the low sensitivity of the procedure, bacteriological culture is not used for routine diagnosis of *M. hyopneumoniae* (Friis, 1975).

The **detection of *M. hyopneumoniae* antigen in lung tissue** can be performed by immunofluorescence (IF) and by immunohistochemistry. Immunofluorescence is a semi-quantitative assay to assess the amount of *M. hyopneumoniae*-organisms in the airways (Kobisch *et al.*, 1978). Commonly used scores range from 0 to 3: 0 (no IF), 1 (limited IF), 2 (moderate IF) and 3 (intense IF) (Vicca *et al.*, 2003). The limitations of this technique include

the laborious and time-consuming procedure, the diagnosis can only be done post-mortem and the semi-quantitative outcome obtained, which does not allow to accurately quantify the load of *M. hyopneumoniae* organisms.

The **detection of *M. hyopneumoniae* DNA** by PCR testing is currently widely used to investigate the potential role of this agent in respiratory disease. The accurate detection level of infection, as well as the relatively rapid and inexpensive characteristic of this method, have favored its establishment in routine diagnostic labs (Thacker and Minion, 2012). PCR assays are suitable for detection of *M. hyopneumoniae* in a wide range of samples, including lung tissue, nasal and trachea-bronchial swabs, as well as broncho-alveolar lavage fluid. Different PCR assays have been described. Nested PCR has an excellent sensitivity, even capable to detect as few as 4 or 5 DNA copies (Stärk *et al.*, 1998a). Recently, a real-time (quantitative) PCR (qPCR) has been developed which allows to quantify the number of DNA copies per ml (Marois *et al.*, 2010). Even the problem of identifying multiple mycoplasmas that grow in culture broth has been solved with the development of a multiplex PCR (Stakenborg *et al.*, 2006a). Although this increased accuracy allows detection of *M. hyopneumoniae* earlier than seroconversion, contamination of the samples should be avoided as this may result in false positive results (Thacker and Minion, 2012).

Serology is commonly used to detect serum **antibodies against *M. hyopneumoniae*** (Calsamiglia *et al.*, 1999; Thacker, 2004) at group or herd level. Commercial ELISAs generally have a good specificity. However, the sensitivity of these assays may be low, ranging from 37 to 49 per cent, which results in a high percentage of false negatives (Thacker, 2004). Consequently, the use of ELISAs is specially recommended for monitoring the *M. hyopneumoniae* status of a population, and, therefore, careful interpretation at individual level is required.

1.5.5. Molecular typing

Molecular typing techniques (*e.g.* pulsed-field gel electrophoresis, amplified fragment length polymorphism, randomly amplified polymorphic DNA, PCR-random fragment length polymorphism, multiple-locus variable number of tandem repeats analysis) have been developed recently and they are mainly used to investigate the diversity of *M. hyopneumoniae* strains (Stakenborg *et al.*, 2005b; 2006b).

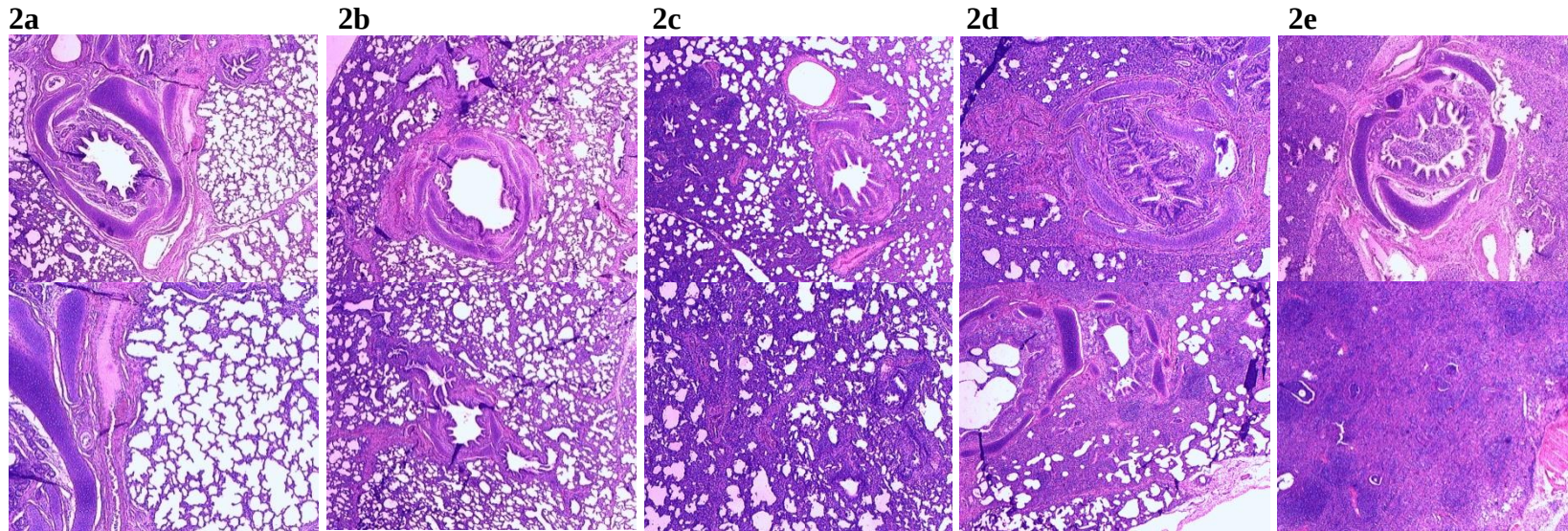


Figure 2. Microscopic scoring system to evaluate the severity of peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation related with *M. hyopneumoniae* induced pneumonia lesions (Morris *et al.*, 1995). The following scores are represented in the picture: **(2a)** score 1 [limited cellular infiltrates (macrophages and lymphocytes) around bronchioles, with airways and alveolar spaces free of cellular exudates]; **(2b)** score 2 (light to moderate infiltrates with mild diffuse cellular exudates into airways); **(2c)** score 3, **(2d)** score 4 and **(2e)** score 5 (mild, moderate and severe lesions characteristic of broncho-interstitial pneumonia, centered around bronchioles but extending to the interstitium, with lymphofollicular infiltration and mixed inflammatory cell exudates). Scores 1 and 2 are considered non *M. hyopneumoniae*-related, whereas scores 3 to 5 are suggestive of *M. hyopneumoniae* infection.

1.6. TREATMENT AND CONTROL OF *MYCOPLASMA HYOPNEUMONIAE* INFECTIONS

The control of *M. hyopneumoniae* infections can be accomplished by optimization of management practices and housing conditions, antimicrobial treatment and vaccination (Maes *et al.*, 2008a).

1.6.1. Optimization of management practices and housing conditions

Improvement of the management practices and the environment of the animals is of crucial importance in the control of *M. hyopneumoniae* infections. Most authors classify non-infectious risk factors in three categories (management, environment and pig factors) (Stärk 2000; Brockmeier *et al.*, 2002; Opriessnig *et al.*, 2011), but it is clear that in practice, many factors may interact with each other. A short description of the main control and preventive measures that reduce the occurrence and transmission of respiratory pathogens is presented in table 1.

Table 1. Control and preventive measures that reduce the occurrence and transmission of respiratory pathogens.

Control and preventive measures				References
Herd characteristics	reduction		increase	
Type of herd	Multi-site systems	vs	Farrow-to-finish	Clarck <i>et al.</i> , 1991; Sibila <i>et al.</i> , 2004a; Maes <i>et al.</i> , 2008a
	All-in/all-out	vs	Continous flow	
	Nursery	vs	Growing-finishing farm	Sibila <i>et al.</i> , 2004a;
Size of the herd	Small	vs	Large	Tuovinen <i>et al.</i> , 1997; Stärk <i>et al.</i> , 1998b
Regional pig density	Low	vs	High	Dee <i>et al.</i> , 2009
Purchase policy	Closed herds	vs	High replacement rate of gilts	Maes <i>et al.</i> , 2000
	Single-source purchase	vs	Multi-source purchase	Stärk <i>et al.</i> , 2000
	Quarantaine	vs	No quarantaine	Amass and Baysinger, 2006
Management practices				
All-in/all-out	Avoid mixing animals of different sources and ages in the same airspace			Clark <i>et al.</i> , 1991
Early weaning	< 3 weeks of age			Maes <i>et al.</i> , 2008a;
Parity segregation	Gilts and their offspring separated from the sows until they reach their second gestation			Hoy <i>et al.</i> , 1986; Joo, 2003.
McREBEL (Management Changes to Reduce Exposure to Bacteria to Eliminate Losses)	Reduction of cross-fostering			McCaw (2000)
	Strict hygiene and biosecurity: - cleaning, disinfection and empty period between batches - changing needles between litters - caretaking and isolation of sick animals, elimination of wasted animals - movement restriction of personnel and material within the herd - control of rodents and birds, and restriction of visits			
Prevention of:	Other diseases by vaccination or medication Mycotoxin contamination of feed			Maes <i>et al.</i> , 2008a Antonissen <i>et al.</i> , 2014
Environmental and housing conditions				
Physical factors	Appropriate stock density (m ² /pig)			Zulovich, 2012
	Sufficient air volume (m ³ /pig)			Flesjå <i>et al.</i> , 1982
	Type of floor, the manure system, the bedding material and the type of feed			Honey and Mcquitty, 1979; Donham 1991
Climate factors	Control of fluctuations of temperature, specially during cold seasons			Maes <i>et al.</i> , 2000; Stärk <i>et al.</i> , 2000; Maes <i>et al.</i> , 2001; Segalés <i>et al.</i> , 2012
	Avoid high relative humidity			
		Efficient ventilation pattern		
Air quality factors	Avoid exposure to aerial pollutants and excessive ammonia			Done, 1991; Wathes <i>et al.</i> , 2004
	Avoid high dust concentrations			Robertson <i>et al.</i> , 1990; Hamilton <i>et al.</i> , 1999; Gonyou <i>et al.</i> , 2006

1.6.2. Antimicrobial treatment

This section summarizes the current knowledge on antimicrobial agents used for the treatment of *M. hyopneumoniae* infections, the route of administration of these antimicrobials, as well as the presence of antimicrobial resistance. Although antimicrobials are capable of controlling *M. hyopneumoniae* infections, complete elimination of the organism from the respiratory tract cannot be achieved by medication (Thacker and Minion, 2012).

1.6.2.1. Antimicrobials

Potentially active antimicrobials against *M. hyopneumoniae* include tetracyclines, macrolides, lincosamides, pleuromutilins, florfenicol, aminoglycosides, aminocyclitols and fluoroquinolones (Vicca, 2005; Maes *et al.*, 2008a; AMCRA, 2013). Only the last two agents have mycoplasmacidal effects (Hannan *et al.*, 1989). To control and treat respiratory disease, tetracyclines (doxycycline), potentiated sulfonamides (sulfadiazine-trimethoprim) and macrolides (tylosin and tilmicosin) are mostly used. Although sulfadiazine-trimethoprim is not effective against *M. hyopneumoniae*, it may be useful for treatment of secondary bacterial infections, often present during enzootic pneumonia. An overview of the main antimicrobials effective against *M. hyopneumoniae* is presented in this section, including pharmacokinetic and pharmacodynamic characteristics, as well as the mode of action and spectrum of activity (Table 2).

1. Tetracyclines

Most of the tetracyclines can be administered *per os*, although intramuscular and intravenous formulations may also be used. Oral bioavailability may be lower when administered in feed, since bivalent cations (calcium, magnesium, iron) have a chelating effect on tetracyclines and, therefore, absorption and activity is reduced (Luthman and Jacobsson, 1983). Oral bioavailability when administered via the drinking water may also be reduced by the water acidity and bivalent cations (iron) of the water pipes (Prescott, 2000b). Some tetracyclines are more lipophilic than other members, such as doxycycline, and therefore diffuse easily through most tissues, biological barriers and cell membranes. The degree of liposolubility largely varies between the different tetracyclines and determines their tissue distribution and elimination rate. Based on the duration of the effect, tetracyclines can be classified in three different groups: short acting (chlortetracycline, tetracycline, oxytetracycline), medium duration (demeclocycline, metacycline) and long acting (doxycycline, minocycline) (Lemos, 2002). Susceptibility testing has demonstrated that some

coliforms (Burch 2013), *Mycoplasma hyopneumoniae* (Inamoto *et al.*, 1994), *A. pleuropneumoniae* (Vanni *et al.*, 2012), *S. suis* (De Jong *et al.*, 2014), *H. parasuis* (De la Fuente *et al.*, 2007) and *P. multocida* (De Jong *et al.*, 2014) have acquired resistance to tetracyclines. Generally, tetracyclines are widely used in the treatment of *M. hyopneumoniae* infections, as well as to treat and prevent atrophic rhinitis and other respiratory infections in pigs caused by *A. pleuropneumoniae* and *P. multocida*.

2. Macrolides

Most of the macrolides can be administered orally or parenterally. They are basic compounds very lipophilic, they are well absorbed from the intestine and have a good tissue distribution (Vicca, 2005). This high liposolubility enables macrolides to diffuse easily through biological barriers, and to reach therapeutic concentrations in most of the tissues, often many times higher than serum concentrations (Lemos, 2002). Once macrolides are distributed through the tissues, they accumulate intracellularly in the lysosomes of the phagocytes (Scoreaux and Shryock, 1998). Acquired resistance to macrolides has been described under field conditions in *M. hyopneumoniae* (Stakenborg *et al.*, 2005a).

Generally, macrolides are recommended to treat *M. hyopneumoniae* infections, as well as pneumonia and respiratory infections caused by other bacteria, such as *A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis*, and *P. multocida*.

3. Lincosamides

Lincosamides are alkaline, lipophilic compounds, which favors absorption through the gastrointestinal tract and diffusion through biological barriers, leading to high tissue concentrations. Lincomycin is indicated in the treatment of *M. hyopneumoniae* infections. Acquired resistance to lincosamides has been described under field conditions in *M. hyopneumoniae* (Stakenborg *et al.*, 2005a).

4. Pleuromutilins

Pleuromutilins present an excellent absorption after oral administration in monogastric mammals (Prescott, 2000c), a high bioavailability (around 90 per cent) and achieve high tissue concentrations in the lung (Burch, 2012). Pleuromutilins are often used to treat PRDC. Tiamulin and valnemulin may be used for the treatment of *M. hyopneumoniae* infections. However, it is better to restrict their use to control *Brachyspira* infections (AMCRA, 2013), since only a few effective antimicrobials are still available for the treatment of swine dysentery due to the increased antimicrobial resistance.

Table 2. Mode of action and spectrum of activity of the main antimicrobials effective against *M. hyopneumoniae*.

Family of antimicrobials	Mode of action	Spectrum	References
Tetracyclines chlortetracycline doxycycline oxytetracycline	Inhibition of protein synthesis by binding reversely to 30S ribosomal subunit, which interferes with association of aminoacyl-tRNA to mRNA-ribosomal complex	Broad-spectrum; bacteriostatic Gram+, Gram-, chlamydiae, mycoplasmas, rickettsiae, protozoa	Aronson, 1980; Riviere <i>et al.</i> , 1991; Prescott, 2000b; Chopra and Roberts, 2001
Macrolides erythromycin tildipirosin, tilmicosin tulathromycin tylosin, tylvalosin	Inhibition of protein synthesis by binding reversely to 50S ribosomal subunit, which interferes with translocation of peptides, hence with growth of peptide chain and results in dissociation of peptidyl-tRNA from ribosome	Bacteriostatic Gram+, selected Gram- (<i>Pasteurella</i> , <i>Mannheimia</i> , <i>Leptospira</i> , <i>Campylobacter</i> , <i>Actinobacillus</i>), some anaerobes and <i>Mycoplasma</i> spp.	Adams, 2001; Vester and Douthwaite, 2001; Vicca, 2005
Lincosamides lincomycin	Inhibition of protein synthesis <i>cfr. Macrolides</i>	Bacteriostatic. Gram+ and anaerobes, but less effective against Gram- and mycoplasmas than macrolides	Prescott, 2000c
Pleuromutilins tiamulin valnemulin	Inhibition of protein synthesis <i>cfr. Macrolides</i>	Bacteriostatic. Gram+, anaerobes and mycoplasmas, but less effective against Gram- than macrolides. Tiamulin: active against Gram- (<i>Actinobacillus</i> , <i>Bordetella</i> , <i>Brachyspira</i> , <i>Haemophilus</i> , <i>Mycoplasma</i> and <i>Pasteurella</i>).	Weisblum, 1998; Aitken <i>et al.</i> , 1999; Vester and Douthwaite, 2001; Lemos, 2002
Amphenicols florfenicol	Inhibition of protein synthesis <i>cfr. Macrolides</i>	Broad-spectrum; bacteriostatic Gram+, Gram-, chlamydiae, mycoplasmas, rickettsiae and anaerobes	Cannon <i>et al.</i> , 1990; Sams, 1994; Priebe and Schwarz, 2003; Shin <i>et al.</i> , 2005
Aminoglycosides apramycin, gentamicin, neomycin, streptomycin Aminocyclitols spectinomycin	Inhibition of protein synthesis by binding irreversibly 30S ribosomal subunit and blocking tRNA translation. Translation fidelity is decreased by disruption of translocation step of protein synthesis and by induction of translation errors	Broad-spectrum; bactericidal Some Gram + (<i>Mycobacteria</i> , <i>Staphylococcus</i> spp.), many aerobic Gram- (<i>Spirochetes</i>) and mycoplasmas	Calvert <i>et al.</i> , 1985; Brown, 1988; Malik <i>et al.</i> , 1994; Riviere and Spoo, 1995; Kotra <i>et al.</i> , 2000; Prescott, 2000a; Lemos, 2002;
Fluoroquinolones danofloxacin enrofloxacin flumequine marbofloxacin	Abolition DNA supercoiling and replication by inhibition of the topoisomerase II, DNA gyrase and topoisomerase IV	Broad-spectrum; bactericidal Some Gram+, most Gram-, chlamydiae, mycobacteria and mycoplasmas	Arai <i>et al.</i> , 1992; Roberts, 1992; Brown, 1996; Hannan <i>et al.</i> , 1997; Papich and Riviere, 2001; Lees and Shojaee AliAbadi, 2002

5. Florfenicol

Florfenicol can be administered orally or parenterally (Liu *et al.*, 2003; Ciprián *et al.*, 2012). This lipophilic drug has a wide tissue distribution. Immediately after intramuscular administration, high initial blood levels are reached, leading to a quick initial response. Duration of therapeutic plasma level may last more than 53 h after intramuscular administration (Liu *et al.*, 2003).

The use of florfenicol is indicated for the treatment of bacterial pneumonia and associated respiratory infections caused by *A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis*, *M. hyopneumoniae*, *M. hyosynoviae*, *P. multocida*, *Salmonella enterica* and *S. suis* (Burch, 2012).

6. Aminoglycosides and aminocyclitols

Aminoglycosides can be administered orally or parenterally. Due to their low lipophilic degree, diffusion through cell membranes is limited and distribution impaired (Giroux *et al.*, 1995). They are rapidly and well absorbed from intramuscular and subcutaneous routes of administration (Hammond, 1953; Brown and Riviere, 1991). When administered parenterally, aminoglycosides easily penetrate in lung parenchyma and bronchial secretions (Saux *et al.*, 1986; Goldstein *et al.*, 2002). Therefore, they are specially indicated in the treatment of *M. hyopneumoniae* infections. However, they are very poorly absorbed after oral administration (Brown and Riviere, 1991). Therefore, the oral route is mainly used to treat enteric infections, and parenteral treatment for other infections.

7. Fluoroquinolones

Fluoroquinolones can be administered via oral, intramuscular or subcutaneous route. Fluoroquinolones are strongly lipophilic and therefore a high tissue distribution is reached with good penetration through biological barriers. In monogastric animals, orally administered fluoroquinolones are absorbed quickly and completely (80-100 per cent) (Inui *et al.*, 1998) and maximum plasma concentrations are reached one hour after intake. Oral absorption is high, but it can be affected by divalent and trivalent cations. Absorption from parenteral administration is rapid and nearly complete (Cabanès *et al.*, 1992; Gavrielli *et al.*, 1995; Kaartinen *et al.*, 1995; Brown, 1996; Mengozzi *et al.*, 1996; Nielsen and Gyrd-Hansen, 1997; Richez *et al.*, 1997; Bailey *et al.*, 1998). They achieve concentrations that are at least as high as plasma in most tissues (Papich and Riviere, 2001) and are rapidly accumulated in macrophages and neutrophils.

Fluoroquinolones can be utilized for the treatment of *M. hyopneumoniae*, as well as other respiratory infections (*P. multocida*).

1.6.2.2. Antimicrobial use and routes of administration

The intensification of swine production in the past years has increased the use of antimicrobials to maintain pig health. Nowadays, the use of antimicrobial remains necessary to control respiratory disease (Mateu and Martin, 2001; Schwarz *et al.*, 2001; McEwen and Fedorka-Cray, 2002; Timmerman *et al.*, 2006; Maes *et al.*, 2008a; Callens *et al.*, 2012).

Based on the objective of the therapeutic medication, antimicrobial use can be divided in three categories: treatment, metaphylactic, prophylactic. Metaphylaxis is defined as the mass medication of a group of animals when some of these animals are clinically diseased while others are subclinically infected (Vicca, 2005). Prophylaxis is defined as the mass medication of a group of animals to prevent a possible clinical outbreak during high-risk periods for disease (Vicca, 2005). The high-risk period of *M. hyopneumoniae* infections occurrence under European production conditions is known to be after transfer of animals to the finishing facilities (10 weeks of age) (Léon *et al.*, 2001). A recent study in Belgian pig herds (Callens *et al.*, 2012) demonstrated that antimicrobials are frequently utilized therapeutically but also metaphylactically or prophylactically. This study illustrated a 7 and 93 per cent of metaphylactic and prophylactic use, respectively, in Belgian pig herds. However, when compared with a similar previous report (Timmerman *et al.*, 2006) (metaphylactic: 44 per cent; prophylactic: 56 per cent), an increased number of prophylactic group treatments and a drastic decrease of the portion of metaphylactic group treatments to the total number of group treatments were observed along the time. These studies have also described an increased number of treatment incidents in 2010 when compared with 2006 in Belgium. Therefore, these results clearly show the need for a reduction of group level prophylactic antimicrobial use (Callens *et al.*, 2012). It also further confirms previous observation which indicated that in modern pig production, animals are mostly treated as a group and not as single individuals (Vicca, 2005; Timmerman *et al.*, 2006; Burch, 2012).

Antimicrobial **group treatments** are predominantly applied via in-feed or in-water medication because oral administration is less laborious and stressful compared to parenteral administration. Oral administration is the most common method of administering antimicrobials at group level. It can be quantified as treatment incidences (TI) based on the animal daily dose pig (ADDpig: average maintenance dose per day per kg pig of a drug used for its main indication) and the used daily dose pig (UDDpig: administered dose per day per

kg pig of a drug) (Callens *et al.*, 2012). Also parenteral treatment can be quantified in this way. It has been shown that oral administrations are mostly underdosed (Timmerman *et al.*, 2006).

It is generally accepted that *in-feed medication* is the most common method of administering antimicrobials at group level. However, it presents various disadvantages, which may be common to both (in-feed and via drinking water) oral administrations (Table 3). First, a proportion of the feed provided to the pigs is wasted and not consumed by them. Gonyou and Lou (1998) and Van Heugten and Van Kempen (1999) showed that wasted feed can range from 5 to 6 per cent. Therefore, to avoid underdosing, the dosage of antimicrobial should be calculated based on real feed intake, and not on total feed provided (Gottlob *et al.*, 2007). Second, the absorption and metabolism of some antimicrobials agents depend on the pH of the stomach and the solubility of the agent. Therefore, a perfect knowledge of the pharmacokinetic properties is required for the selection of the correct antimicrobial. Third, in-feed medication is not recommended for the treatment of acute infections (*e.g.* *A. pleuropneumoniae*), where a quick administration is of crucial importance for the success of the therapy (Pijpers *et al.*, 1990; Henry and Apley, 1999; Friendship, 2000). However, it is especially suited for endemic or chronic diseases, such as enzootic pneumonia. Fourth, it appears that a not depreciable amount of medication is wasted or not ideally administered, since not only sick animals, but also healthy pigs receive medication. This can be justified by the intention to treat also penmates that are at-risk to become infected, especially in herds with a high stocking density.

Antimicrobial group treatment can also be implemented via *drinking water*. Currently, administration of antimicrobials given as soluble formulations in the drinking water is becoming more popular. The development of more reliable dosing/water-proportioner machines has favored this increased use. It is especially recommended for the treatment of acute diseases. Urgent group treatment can be done in a quick way and sick pigs often continue to drink, even when they refuse feed. An important limitation is the antimicrobial wastage due to water disappearance. Water disappearance is defined as the overall usage of water, including water intake and wastage. Water wastage is mainly caused by either the type of drinking system and/or playing with the drinking nipple both intentionally (out of boredom) or unintentionally (highly dense stocked pens) (Brumm and Heemstra, 2000). For example, nipple drinkers waste 50 per cent more than bowl drinkers and nipple drinkers that are activated from any angle waste more than the ones that are activated from a forward angle (Brumm and Heemstra, 2000; Gottlob *et al.*, 2007). Water intake may also be largely

influenced by the season. Therefore, appropriate follow-up of water usage efficiency is a prerequisite for achieving correct therapeutic doses (Henry and Apley, 1999).

Group treatment can also be applied by parenteral administration. Advantages and disadvantages of in-feed, drinking water and the parenteral route of administration are presented in table 3. Callens *et al.* (2012) have illustrated a change in the relative importance of the different routes of administration of group treatments in Belgian pig herds, when compared with 2003 (Timmerman *et al.*, 2006). The oral group treatments appeared to have been replaced by long-acting injectable group treatments. Injectable group treatments are mainly used in suckling pigs, and according to Timmerman *et al.* (2006) and Callens *et al.* (2012), overdosing often takes place.

Individual treatments are usually applied by parenteral administration. Data from Callens *et al.* (2012) revealed that in most of the Belgian herds antimicrobials at individual level were administered. Individual treatments are frequently practiced in pigs suffering from acute disease by parenteral injection during onset and first days of disease. It often results in a good response against the disease, especially when acute outbreaks of respiratory disease occur and appetite of sick animals is diminished (Pijpers, 1990; Henry and Apley, 1999).

The control of the *M. hyopneumoniae* infections by group medication can be accomplished by **strategic administration** of antimicrobials. It is considered as a prophylactic practice in swine (Karriker *et al.*, 2012). It is usually applied by oral administration. Strategic medication may reduce the consequences of the disease and the infection load, but it does not prevent pigs from becoming infected with *M. hyopneumoniae* (Thacker *et al.*, 2006). In addition, the symptoms may reappear after cessation of the therapy. Strategic medication during extended periods of time is not recommended. The risk for antimicrobial residues in pig carcasses at slaughter and the development of antimicrobial resistance in pathogens and bacteria belonging to the normal microbiota are major concerns in Europe (Maes *et al.*, 2008a). Currently, several countries of the EU (Denmark, Sweden, The Netherlands, Germany) are running different programs at national level to monitor and reduce the use of antimicrobials in livestock (Callens *et al.*, 2012).

Table 3. Advantages, disadvantages and reasons of failure to consider when using in-feed, drinking water and parenteral medication (Burch, 2013).

	In-Feed	Drinking water	Parenteral
Indication	Enzootic/chronic	Chronic/acute	Acute
Target population	Mass medication	Mass medication	Individual treatment
Dose accuracy	Low	Variable	High
Application to selective groups	Difficult (healthy pigs treated)	Intermediate	Easy (only sick pigs treated)
Implementation	Slow	Fast	Fast
Labor	Low	Low	High
Cost	Relatively high	Low	High
Risk of cross-contamination	High	Low/Intermediate	Low
Failure due to ...			
Lack of appetite	High in sick animals	NA	NA
High seasonal temperatures	Low consumption	High consumption/ wastage	Stress inflict
Wastage	High	High	Fail to inject
Accuracy of dosification systems	Intermediate	Intermediate	Low
Stress during administration	NA	NA	Possible
Antimicrobial drug stability to gastric pH	Variable absorption	Variable absorption	NA
Gastric emptying and interaction with feed	Variable absorption	Variable absorption	NA
Reduction of oral bioavailability	Variable (<i>e.g.</i> Tetracyclines: presence of bivalent cations)	Variable (<i>e.g.</i> Tetracyclines: presence of bivalent cations and water acidity)	NA
Homogeneity of antimicrobial drug in vehicle	Variable	Higher compared to in-feed	NA
Stability of antimicrobial drug in vehicle	Higher compared to in-water	Variable	NA

NA: not applicable

1.6.2.3. Efficacy of antimicrobials against *M. hyopneumoniae* infections under experimental and field conditions

Various studies have been performed in the past years to assess the efficacy of commercial antimicrobials used for the control and treatment of *M. hyopneumoniae* infections. A summary of the principal results obtained from these studies published in peer-reviewed journals is given in tables 4 and 5. Since these studies were conducted under different conditions and many variables were included, comparison of results is complicated. However, it can be concluded that for the majority of antimicrobials tested, performance losses, clinical signs and lung lesions were reduced in treated animals. Nevertheless, *M. hyopneumoniae* could still be isolated from treated animals.

Table 4. The effect of different antibiotic regimens for treatment of experimental infections with *Mycoplasma hyopneumoniae*.

References	Antibiotic and dosage	Scheme	Effects*
Hannan and Goodwin, 1990	6-chloro analogue of Norfloxacin 400 or 200 ppm Norfloxacin 100 ppm in-feed	during 3 w starting 1 m after infection	improvement of ADG and FCR improvement of LL by 6-chloro analogue at 400 ppm only
Schuller <i>et al.</i> , 1977	Tiamulin 100 or 200 ppm in-feed	during 10 d starting 2 d before infection	improvement of ADG and FCR LL were not prevented Mh could still be isolated
Goodwin, 1979	Tiamulin 50 mg/kg in-feed	during 10 d starting 1 m after infection	improvement of ADG, CS and LL Mh could still be isolated
Kobisch and Sibelle, 1982	Tiamulin 240 ppm in-feed	during 10 d starting 10 d after infection	improvement of ADG, FCR, CS, MLL and mLL
Hsu <i>et al.</i> , 1983	Tiamulin 10, 20, 30 ppm in-feed	during 28 d starting 14 d after infection	improvement of ADG and FCR no beneficial effect on CS and LL
Ross and Cox, 1988	Tiamulin 60, 120, 180 ppm in-water	during 10 d starting 11 d after infection	no beneficial effect on ADG, FCR, CS, MLL and mLL
Hannan <i>et al.</i> , 1982	Tylosin tartrate 50 mg/kg combined with Tiamulin 10 mg/kg in-water	during 10 d starting 14 d after infection (with lung homogenate)	improvement of MLL Mh could still be isolated less secondary bacteria
Clarck <i>et al.</i> , 1998	Tilmicosin 363 ppm in-feed	during 21 d starting 7 d before infection	improvement of CS no beneficial effect on ADG and LL
Thacker <i>et al.</i> , 2006	Chlortetracycline 500 ppm in-feed	during 14 d starting 3 d before or 10 d after infection	3 d before infection: improvement of CS, LL both regimens: reduction of Mh organisms, but it could still be isolated
Vicca <i>et al.</i> , 2005	Tylosin tartrate 100 mg/kg in-feed	during 21 d starting 12 d after infection	no beneficial effect on ADG improvement of CS and MLL Mh could still be isolated
Ciprián <i>et al.</i> , 2012	Florfenicol 20 ppm in-feed	during 35 d starting the day of infection	improvement of ADG and LL Mh could still be isolated
McKelvie <i>et al.</i> , 2005	Tulathromycin 2.5 mg/kg IM Enrofloxacin 5 mg/kg IM	during 1 or 3 d starting 5 d after infection	Tulathromycin: improvement weight gain, CS, LL Enrofloxacin: improvement of CS, LL
Le Carrou <i>et al.</i> , 2006	Marbofloxacin 1 or 2 mg/kg IM	during 3 d starting 27 and 4 d after infection	no beneficial effect on ADG, MLL Mh could still be isolated

* Adapted from Vicca (2005); IM: intramuscular; d: day(s); w: week(s); m: month(s); ADG: average daily gain; FCR: feed conversion ratio; LL: lung lesions; CS: clinical signs; MLL: macroscopic LL; mLL: microscopic LL;

Table 5. The effect of different antibiotic regimens for treatment of infections in herds clinically affected by enzootic pneumonia.

References	Antibiotic and dosage	Scheme	Effects*
Frank, 1989	Enrofloxacin 5 mg/kg <i>Per os</i> + IM + IM	Per os: during 3 w starting after birth IM: during 3 w every 3 d starting after weaning IM: during 1 w every 2 d starting immediately	prevention of CS
Ganter, 1995	Chlortetracycline 800 ppm in-feed	during 3 w	improvement of CS and oxygen saturation
Lukert and Mulkey, 1982	Lincomycin 5 mg/kg IM	at 1, 2, 3, 39, 40, 41 d	improvement of ADG, FCR and CS
Martineau <i>et al.</i> , 1980	Tiamulin 200 ppm in-feed	during 10 d in growing unit	improvement of ADG, CS, MLL and mortality rate
Burch, 1984	Tiamulin 30 ppm in-feed	during 8 w in pigs from 30 to 70 kg	improvement of ADG, FCR no beneficial effect on MLL
Burch <i>et al.</i> , 1986	Tiamulin 100 ppm + Chlortetracycline 300 ppm or Chlortetracycline 300 ppm alone in-feed	during 7 d	Tiamulin + Chlortetracycline: improvement of ADG and FCR, but no beneficial effect on MLL Chlortetracycline: improvement of ADG
Kunesh, 1981	Tylosin 4 mg/kg or Lincomycin 5 mg/kg IM	during 3 d after birth or and for 3 d at weaning	Tylosin: improvement of ADG no beneficial effect on FCR and CS for both antibiotics

References	Antibiotic and dosage	Scheme	Effects*
Binder <i>et al.</i> , 1993	Tilmicosin 300 ppm in-feed	during 9 or 14 d	improvement of ADG, CS and less secondary bacteria
LeGrand and Kobisch, 1996	Tiamulin 200 ppm + Chlortetracycline 600 ppm in-feed	pulse medication: 2 d treatment/2 w during fattening period	lower prevalence on MLL no beneficial effect on severity of MLL
Kavanagh, 1994	Tiamulin 30 ppm + Oxytetracycline 300 ppm in-feed	pulse medication: 2 or 3 d treatment/w during fattening period	improvement of ADG and FCR lower prevalence and severity of MLL financial benefit
Jouglar <i>et al.</i> , 1993	Tiamulin 40 ppm + Oxytetracycline 300 ppm in-feed	pulse medication: 2 d treatment/w during fattening period	improvement of ADG and mortality rate no beneficial effect on FCR and severity of LL
Bousquet <i>et al.</i> , 1998	Marbofloxacin 1 or 2 mg/kg IM	during 3 d starting 27 and 4 d after infection	no beneficial effect on ADG, MLL Mh could still be isolated
Nanjani <i>et al.</i> , 2005	Tulathromycin 2.5 mg/kg IM Tiamulin 15mg/kg IM Florfenicol 15mg/kg IM	at 0 d at 0, 1, 2 d at 0, 2 d	similar beneficial effect for all 3 antimicrobials on ADG
Nutsch <i>et al.</i> , 2005	Tulathromycin 2.5 mg/kg IM Ceftiofur 3mg/kg IM	at 0 d at 0, 1, 2 d	similar beneficial effect for both antimicrobials on cure and mortality rate
Mateusen <i>et al.</i> , 2001	Vaccination (two-shot) Tilmicosin 200 ppm in-feed	at 4 and 22 d of age during 3 w (34-55 d) and 2 w (77-98 d)	similar improvement of ADG and FCR, CS, MLL
Mateusen <i>et al.</i> , 2002	Vaccination (two-shot) Lincomycin 200 ppm in-feed Lincomycin 200 ppm + Vaccination	at 4 and 28 d of age during 3 w in fattening period	similar improvement of ADG and FCR, CS, MLL

* Adapted from Vicca (2005); IM: intramuscular; d: day(s); w: week(s); m: month(s); ADG: average daily gain; FCR: feed conversion ratio; LL: lung lesions; CS: clinical signs; MLL: macroscopic LL; mLL: microscopic LL;

1.6.2.4. Antimicrobial susceptibility of *M. hyopneumoniae*

Few data is available concerning the antimicrobial susceptibility and resistance for *M. hyopneumoniae*. Additionally, the fastidious growth of *M. hyopneumoniae* and its time consuming isolation, complicate the consistency of susceptibility testing and the interpretation of results.

Standard broth and agar dilution methods used for susceptibility testing of other bacteria have been adapted for use with mycoplasmas. The 'International Research Programme on Comparative Mycoplasmology (IRPCM)' suggested the adoption of 'Guidelines and recommendations for antimicrobial minimum inhibitory concentration testing against veterinary mycoplasma species' (Hannan, 2000).

Nowadays, both broth dilution and microbroth dilution techniques have been adapted for mycoplasmas and are the most widely used. The test consists on the inoculation of a standardized number of *Mycoplasma*-organisms in 96-well microtiter plates. These 96-well microtiter plates are prepared with serial doubling concentrations of antimicrobial agents and then, a mycoplasmal medium is added to these broth dilutions. The mycoplasmal medium contains a constant number of microorganisms and a substrate (glucose, arginin or urea) which is metabolized by mycoplasmas. The degradation of this medium results in a pH change visible as color change, which implies mycoplasma growth. The presence of increasing antibiotic concentrations permits to determine the lowest antimicrobial concentration that inhibits mycoplasma growth. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that prevents a color change at the same moment that the color in the control without antibiotic has changed, hence the lowest concentration that prevents mycoplasma growth (Vicca, 2005). The study of antimicrobial susceptibility by means of MIC determinations for *M. hyopneumoniae* has various limitations. Bacterial contamination may occur, resulting in turbidity or color change in broth controls. Since MIC determination of *M. hyopneumoniae* is time consuming and may last up to 14 days, MICs of unstable antibiotics (chlortetracycline, valnemulin) are unreliable. In addition, there are no specific clinical breakpoints for mycoplasmas.

Antimicrobial resistance in *Mycoplasma* spp. may be categorized in two types: intrinsic or acquired resistance. Intrinsic, innate or natural resistance may be defined as the relative insensitivity of all members of a bacterial species or genus against an antimicrobial agent (Vicca, 2005). Members of the class *Mollicutes* lack a cell wall and are therefore resistant to all β -lactam antibiotics, such as penicillins and cephalosporins, as well as antibiotics targeting

the cell wall, like glycopeptides. Additionally, mycoplasmas are also resistant to polymyxins, sulfonamides, trimethoprim, naladixic acid and rifampin. Within *Mycoplasma* species, macrolides present specific patterns of innate resistance depending on the structure of the lactone ring. The 14-membered lactone ring macrolides are ineffective against *M. hyopneumoniae*, *M. flocculare*, *M. bovis*, *M. pulmonis* and *M. fermentans* (Williams, 1978; Tanner *et al.*, 1993; Chambaud *et al.*, 2001; Béb  ar and B  b  ar, 2002; Francoz *et al.*, 2005), whereas *M. pneumoniae* and *M. genitalium* are susceptible to this subgroup of macrolides (B  b  ar *et al.*, 2000; Kenny and Cartwright, 2001). In general terms, there are three main biochemical mechanisms of both intrinsic and acquired antimicrobial resistance in mycoplasmas, *e.g.* active efflux mechanisms, enzymatic modification of the drug or alteration in the drug target site. A complete overview of the biochemical mechanisms involved in the antimicrobial acquired resistance for *Mycoplasma* spp. was published by Vicca (2005).

In vitro susceptibility of *M. hyopneumoniae* to antimicrobials used in pig veterinary medicine has been studied. Apart of intrinsic antimicrobial resistance, acquired antimicrobial resistance of *M. hyopneumoniae* has also been documented. Inconsistent results have been reported in susceptibility testing of tetracyclines (Williams, 1978; Etheridge *et al.*, 1979; Yamamoto and Koshimizu, 1984). Shortly afterwards, Inamoto *et al.* (1994) reported acquired antimicrobial resistance to tetracyclines (chlortetracycline and oxytetracycline) occurring in *M. hyopneumoniae* field strains isolated in Japan between 1970 and 1990. More recently also acquired antimicrobial resistance to macrolides (tylosin, tilmicosin), lincosamides (lincomycin) and fluoroquinolones (enrofloxacin, flumequine) (Vicca *et al.*, 2004) has been documented.

Despite some reports on resistance against tetracyclines, macrolides, lincosamides and fluoroquinolones in *M. hyopneumoniae* (Stakenborg *et al.*, 2005a; Le Carrou *et al.*, 2006; Vicca *et al.*, 2007), resistance against other antimicrobials has not yet been detected. This indicates that antimicrobial resistance does not constitute a major problem for treatment of *M. hyopneumoniae* infections (Vicca *et al.*, 2004; Maes *et al.*, 2008a).

1.6.3. Vaccination against *Mycoplasma hyopneumoniae*

1.6.3.1. Commercial vaccines

Vaccination against *M. hyopneumoniae* is practiced in most of the swine producing countries, being in some cases applied in more than 70 per cent of the herds (Maes *et al.*, 2008a). Vaccination with commercial bacterins is considered as an important and effective tool to control *M. hyopneumoniae* infections.

Commercial bacterins, consisting of inactivated, adjuvanted whole-cell preparations, have been commonly demonstrated efficacious to control the disease. The benefit of vaccination is based on reduction of losses caused by poor performance (average daily gain, ADG; 2-8 per cent), poor feed efficiency (feed conversion ratio; 2-5 per cent) and mortality (Maes *et al.*, 2008a). A shorter time to reach market weight, decreased clinical signs and lung lesions and lower costs for antimicrobial medication and vaccination are contributors to this economic benefit (Dohoo and Montgomery 1996; Jensen *et al.*, 2002; Maes *et al.*, 2003; Maes *et al.*, 2008a). A summary of experiments conducted under field conditions and the benefits observed after vaccination is presented in table 6.

Despite all these advantages, commercial vaccines present various limitations. Thacker *et al.* (2000) suggested that current bacterins provided only partial protection and do not prevent colonization of the respiratory tract by *M. hyopneumoniae*. However, Sibila *et al.* (2007b) also demonstrated that vaccination may reduce the infection level in a herd. This corroborates previous observations from Haesebrouck *et al.* (2004), which indicated that maximum beneficial effects of vaccination are reached several months after the initiation of vaccination. Recent studies demonstrated that vaccination significantly reduced the number of *M. hyopneumoniae* organisms in the lungs of experimentally infected pigs (Vranckx *et al.*, 2012b), but the fact that *M. hyopneumoniae* DNA could still be detected by PCR confirmed previous results from experimental and field transmission studies (Meyns *et al.*, 2006, Villarreal *et al.*, 2011). These studies showed a limited reduction in transmission in vaccinated pigs. Therefore, all these findings suggest that, similarly to antimicrobials, vaccination alone does not prevent infection, and it is not able to eliminate *M. hyopneumoniae* from infected pig herds.

During the past 15 years, few studies have investigated the immune mechanisms involved in induction of protection after vaccination with bacterins. All these studies indicated that both systemic and mucosal immune responses play a central role in the control of *M. hyopneumoniae* infections. However, the exact mechanism of protection conferred by

vaccination against *M. hyopneumoniae* is not yet fully understood. Thacker *et al.* (1998) compared the level of protection of four different commercial bacterins induced after experimental challenge. All four induced the production of specific antibodies in serum, but only a partial protection against clinical signs was conferred. Djordjevic *et al.* (1997) further confirmed this observation. A reduction in pneumonia was observed after vaccination, but antibody concentrations (in serum and respiratory tract washings) were not correlated with protection from the disease. Nowadays it is generally accepted that the presence and concentration of serum antibodies are not correlated with protection. Therefore, this is not suitable for the evaluation of protective immunity (Maes *et al.*, 2008a; Marchioro *et al.*, 2013).

Because *M. hyopneumoniae* is a mucosal pathogen which mainly adheres to the cilia of the epithelial cells from the respiratory tract, local *M. hyopneumoniae*-specific antibodies seem to play an important role in protection. Thacker *et al.* (2000) suggested that both local mucosal antibodies and systemic cell-mediated immunity responses are important for protection. These observations were confirmed by Marchioro *et al.* (2013). These authors illustrated the induction of both local and systemic immune responses involving both specific antibodies and cellular immunity following vaccination against *M. hyopneumoniae* with a commercially available bacterin.

An inconsistent immune response and protection conferred by different commercial vaccines has been reported (Thacker *et al.*, 1998). This variability may be due to several factors, such as different adjuvants included by commercial vaccines and possible antigenic differences (*e.g.* absence of specific epitopes) between the vaccine strain and the strains circulating in the field (Marchioro *et al.*, 2013).

1.6.3.2. Strategies of vaccination

Many studies have been conducted in order to investigate the efficacy of the commercial bacterins under field conditions (Jensen *et al.*, 2002). However, to decide whether vaccination in a specific herd should be implemented has to be based not only on efficacy parameters, but also on a cost-benefit analysis (Maes *et al.*, 2003). Type of the herd, production system, infection pattern, health status of the population at risk, as well as easy implementation at practical level are main variables playing a role in the design of a successful vaccination strategy.

First, it has to be decided which **population at risk** should be vaccinated. In practice in Europe, sow vaccination is rarely practiced, whereas the main strategy of *M. hyopneumoniae* control is vaccination of piglets before or at weaning (Haesebrouck *et al.*, 2004).

Vaccination of sows ensures immunization of the newborn piglets through the passage of maternal derived antibodies via colostrum. However, colostral immunity does not prevent colonization of the piglets with *M. hyopneumoniae* (Thacker *et al.*, 2000; Sibila *et al.*, 2008). The main objective of vaccinating sows is to decrease vertical transmission from the dam to the off-spring via nose-to-nose contact (Ruiz *et al.*, 2003).

Vaccination of gilts before entering into the sow herd may avoid destabilization of the herd immunity (Bargen, 2004).

Vaccination of suckling piglets (early vaccination; <4 weeks of age) is often practiced in single-site herds, where early infections are common (Maes *et al.*, 2008a). Because *M. hyopneumoniae* infections can take place in piglets already during the first (from 1 to 3) weeks of life (Calsamiglia and Pijoan 2000, Ruiz *et al.*, 2003, Fano *et al.*, 2007, Sibila *et al.*, 2007a, Nathues *et al.*, 2010, Villarreal *et al.*, 2010, 2011, Vranckx *et al.*, 2012a, Fablet *et al.*, 2012a), and because vaccination is most likely effective if active immunity can be established before the exposure to the pathogen, vaccination is commonly applied in suckling piglets. When compared with weaned piglets, suckling piglets are less infected with pathogens, such as PRRSV and PCV-2, which may cause problems after weaning and interfere with the establishment of a protective immune response to *M. hyopneumoniae* vaccination (Maes *et al.*, 2008a). A limitation of early vaccination is the presence of maternal antibodies and its possible interference with vaccine efficacy (grosse Beilage *et al.*, 2005; Martelli *et al.*, 2006; Bandrick *et al.*, 2008). However, the majority of the pig herds in Europe vaccinate piglets in the presence of maternal antibodies and the efficacy is apparently not affected (Martelli *et al.*, 2006; Maes *et al.*, 2008a).

Vaccination of weaners/growers/early fattening pigs (late vaccination; between 4 and 10 weeks) is frequently applied in multi-site systems, where late infections are more common (Maes *et al.*, 2008a). Late vaccination may evade the possibility of interference with maternally derived antibodies. However, it may elongate the age-window between decline of maternal immunity and age of infection, leading to a higher risk of infection before immunization. In addition, nursery pigs may already be infected with *M. hyopneumoniae*, since the onset of infection may vary between herds and also within a herd among successive batches (Sibila *et al.*, 2004b, 2007b, Fano *et al.*, 2007, Segalés *et al.*, 2012).

Vaccination programs have become even more complicated with the introduction of **single-dose** formulations in addition to **traditional two-dose** formulations. The first commercially available bacterins indeed required two intramuscular injections and were demonstrated efficacious to control the disease. However, with the appearance of single-dose vaccines, which were confirmed as equally efficacious as the two-dose formulations (Kriakis *et al.*, 2001; Dawson *et al.*, 2002), the newer bacterins became more popular. This was mainly due to the reduced labor costs, easy implementation at farm level, less stress inflicted to the pigs, while a similar protection is conferred when compared with two-shot vaccines (Roof *et al.*, 2001; Roof *et al.*, 2002; Alexopoulos *et al.*, 2004; Lillie *et al.*, 2004; Baccaro *et al.*, 2006; Greiner *et al.*, 2011). Despite the acceptable efficacy of single-dose vaccines, there are still some situations in which two-dose formulations may be indicated (Yeske *et al.*, 2001). These situations include critical periods (pigs placed in fall months) and production systems (multi-source, multi-age, continuous flow) with serious *M. hyopneumoniae* challenges. It is also recommended in large herds with unstable situation to PRRSV, as well as when vaccination compliance is doubtful (Yeske *et al.*, 2001).

Variation in protection between different bacterins may be associated with the different **adjuvants**. Although all commercial bacterins are made from inactivated *M. hyopneumoniae* cells, they differ in the type of adjuvant (aluminium hydroxide, carbopol, mineral oil or biodegradable oil) and the solution included. Vaccines containing carbopol or aluminium hydroxide are produced as water based suspensions, whereas the oil adjuvant vaccines are produced as different emulsions. It is generally accepted that aqueous-based adjuvants produce a lesser stimulation of the immune system, and, consequently re-vaccination (two-shot) is needed. Oil-based adjuvants are more reactive and often take a longer time to be processed. Therefore, they provide a prolonged release of the antigen and a better stimulation of the immune system. Generally speaking, bacterins containing oil-based adjuvants are capable to stimulate the immune system over time, mimicking conventional two-shot vaccines. Oil-based adjuvants can be divided in two types: oil in water emulsions and water in oil emulsions. Oil in water emulsions present relatively good immune stimulating properties, but a quick release of the antigen, which may shorten the duration of immune stimulation. In order to achieve a long term protection, commercial vaccines include mineral oil, which may cause significant local reactions. These local side effects may be minimized by water in oil emulsions, which are made of very small droplets of antigenic medium dispersed in biodegradable oil. This formulation based in biodegradable oil ensures an initial fast immune response and a subsequent slow release of the antigen. This provides a smooth

but long lasting stimulation of the immune system. Therefore, the onset of immunity is modulated by the progressive liberation of the antigen after only a few days and this immunity is maintained for a long period, compared to other adjuvant models (Herbach, personal communication, 2005; Aucouturier *et al.*, 2002).

The efficacy of all different commercial vaccines has been widely demonstrated during the last years. Numerous clinical trials both under experimental and field conditions have been carried out. Numerous parameters of comparison have been included. A summary of the experiments carried out in the past is presented in table 6. It includes type of vaccine, doses and age of vaccination, conditions of the experiment, parameters of evaluation, and significant results obtained.

The **route of vaccine administration** has also to be taken into account when designing a vaccination strategy. Traditionally, intramuscular administration has been used. Apart from intramuscular vaccination, the effect of intraperitoneal (Sheldrake *et al.*, 1991), oral (Weng *et al.*, 1992), aerosol (Murphy *et al.*, 1993), intranasal (Shimoji *et al.*, 2003) and intradermal (Jones *et al.*, 2005) vaccination against *M. hyopneumoniae* has also been investigated. Nowadays, intradermal vaccination has arisen as an alternative to intramuscular administration. It has been shown to be as efficacious as the intramuscular route in reducing performance losses and providing a better protection against clinical signs and lung lesions (Tassis *et al.*, 2012). Main advantages of intradermal administration include lower risk of disease transmission through the application (no needles) and the consistent delivery of the vaccine. The lower volume and higher dispersion of the antigen during administration is associated with a total reduction of side effects at the injection site and a decrease of condemnation rate of carcasses at slaughter. Less pain and stress is inflicted to the animals and it is considered to be a safe procedure for the operators. Disadvantages of intradermal vaccination include the high cost for purchasing and maintaining the device for the ID application, and the fact that training of the person using it is needed.

The use of combination vaccines has increased in the last years. Combination vaccines are those that contain antigens from different pathogens in the same formulation. Combined vaccination may maximize vaccine benefits, while minimizing costs, labor and the stress inflicted to the animals (Drexler *et al.*, 2010). Although they are widely used in U.S., they lack popularity in Europe. A few studies have been carried out to investigate the effect of different combinations of vaccines against different pathogens [PRRSV and *M. hyopneumoniae* (Drexler *et al.*, 2010); PRRSV, *M. hyopneumoniae* and *A. pleuropneumoniae* (Delisle and Rigaut, 2007); PCV-2 and PRRSV (Bretey *et al.*, 2010);

PCV-2 and *M. hyopneumoniae* (Hayes and Saltzman, 2009)]. These studies showed that vaccines against different pathogens can be combined without compromising the efficacy and safety, when used according to the manufacturer's instructions.

1.6.3.3. Development of new vaccines

Commercial vaccines are whole cell, adjuvanted bacterins (Okada *et al.*, 1999).

Nowadays, the availability of complete genome sequences has contributed to a new approach in vaccine development (Scarselli *et al.*, 2005), called reverse vaccinology (Rappuoli, 2001). The reverse vaccinology approach starts from the genomic sequence and, by computer analysis, predicts those antigens that are most likely to be vaccine candidates. This method may be used for the development of both subunit and DNA vaccines.

The use of recombinant proteins contributed to the development of subunit vaccines. The recombinant subunit vaccines are based on fractions of the organism and are produced using heterologous protein expression systems. These heterologous proteins can be produced in *e.g. Escherichia coli* cells and, once expressed and purified, can be administered to the animals. Specific antigens of *M. hyopneumoniae* include surface proteins (P46, P65, P97), cytosolic proteins (P36) and functional enzymes (L-lactate dehydrogenase, ribonucleotide reductase) (Kim *et al.*, 1990; Strasser *et al.*, 1991; Futo *et al.*, 1995; Zhang *et al.*, 1995; Fagan *et al.*, 1996), and may be considered as potential vaccine candidates.

Recently, several experimental recombinant subunit vaccines have been developed and tested for immune responses in mice (Marchioro *et al.*, 2012) or pigs (Marchioro *et al.*, unpublished). Excellent reviews have been published by Simionatto *et al.* (2013) and Marchioro (2013). Significant immune responses have been demonstrated in mice and pigs immunized with these experimental vaccines. Thus, recombinant subunit vaccines might represent a promising strategy for developing more effective vaccines against *M. hyopneumoniae*.

Table 6. Field studies investigating the effect of vaccination against *M. hyopneumoniae* in pig herds clinically affected with enzootic pneumonia.

Reference	Number of herds and pigs	Commercial vaccine Schedule (age at vaccination in w)	Efficacy parameters					
			Increase in ADG g/pig/d (%)	FCR %	Pneumonia lesions at slaughter		Mortality	Other variables improved
					prevalence	extent		
Vraa-Andersen and Christensen, 1993	5 herds/ 1500 pigs	Suvaxyn two-shot 1+3 w 4+6 w	+10 (2%) +25 (4%)	-	-49%	Significantly decreased	NI	-
Scheidt <i>et al.</i> , 1994	1 herd/ 150 pigs	Respire two-shot 1+3 w 6+8 w	+60 (8%) +60 (8%)	NI	NI	-6% -8%	-	More feed consumption Less coughing
Dohoo and Montgomery, 1996	1 herd/ 220 pigs	Respire two-shot 3+6 w	-	-	-33%	Significantly decreased	-	Age to reach 80 kg carcass weight: -11d or -2d Carcass weight: +1 kg Less carcasses under 70 kg
LeGrand and Kobisch, 1996	1 herd/ 911 pigs	Stellamune two-shot 1+4 w	+16 (2%)	-	-24%	-1%	-	Age at 100 kg: -2.4d Carcass weight: +0.76 kg Less coughing Meat %: +0.64
Schatzmann <i>et al.</i> , 1996	1 herd/ 120 pigs	Stellamune two-shot 1+3 w	+60 (8%)	-	-10%	Significantly decreased	-	Vaccinated pigs still excrete Mhyo
Diekman <i>et al.</i> , 1999	1 herd/ 216 gilts	Respire two-shot 1+4 w	-20 (-3%)	NI	-10%	-6%	-	-
Okada <i>et al.</i> , 1999	3 herds/ 212 pigs	Mycobuster two-shot 3-to-7 + 7-to-11 w	NI +44 (7%)	NI	-43% -54%	Significantly decreased	-	Age to market weight: -12d

Maes <i>et al.</i> , 1998	5 herds/ 468 pigs	Stellamune two-shot 1+3 w	+25 (4%)	-	-27%	-11%	NI	Age to reach 100kg liveweight: -5d Medication costs/pig: -11% % pigs with healthy lungs: +22%
Maes <i>et al.</i> , 1999	14 herds/ 7000 pigs	Stellamune two-shot 1+3 w	+22 (3%)	-0.1%	-14%	-3%	NI	Age to reach 100kg liveweight: -5d Medication costs/pig: -40% % pigs with healthy lungs: +36% Coughing index: reduction
Kyriakis <i>et al.</i> , 2001	1 herd/ 390 pigs	Hyoresp two vs one-shot 1+4 w 10 w	Significant increase	+13% +5%	-	-2% <1%	-	Significant reduction in medication costs
Dawson <i>et al.</i> , 2002	2 herds/ 1392 pigs	Stellamune one-shot 3-to-5 w	+23 (4%)	-	-	Significantly decreased	-	-
Llopart <i>et al.</i> , 2002	3 herds/ 706 pigs	Mypravac suis two-shot 1+4 w	+82 (12%)	-0.3	-7%	Significantly decreased	NI	-
Moreau <i>et al.</i> , 2004	1 herd/ 59740 pigs	Ingelvac Mhyo one-shot 9 w	+42 (5%)	NI	-	-	-1.5%	-
Baccaro <i>et al.</i> , 2006	1 herd/ 520 pigs	Respire1 vs Ingelvac Mhyo one-shot 4 w	+30 (4%) +40 (5%)	NI	-51% -31%	Significantly decreased	-	-
Villarreal <i>et al.</i> , 2011	1 herd/ 72 pigs	Suvaxyn MH-one one-shot 3 w	+49 (8%)	-	-8%	NI	-	Vaccination does not reduce transmission of <i>M. hyopneumoniae</i>
Tassis <i>et al.</i> , 2012	1 herd/ 1051 pigs	Porcilis-Mhyo ID vs IM One-shot 4 w	+23(4%) +5 (1%)	-	-14% -5%	Significantly decreased	-	Better protection in reducing lung lesions by ID

* Adapted from Maes (1998); IM: intramuscular; ID: intradermal; d: day(s); w: week(s); ADG: average daily gain; FCR: feed conversion ratio; “-“ not applicable; NI: numerical increased

1.6.4. Preventive medication vs vaccination

It is well known that neither vaccination nor preventive medication can prevent adherence of *M. hyopneumoniae* to the ciliated cells of the respiratory tract and infection (Le Grand and Kobisch, 1996). However, the advantages and disadvantages of both strategies may be complementary in the control of *M. hyopneumoniae* infections (Table 7). Antimicrobial medication has a flexible and immediate effect whereas the effect of vaccination will be only evident in a long term and at herd level, when practiced for at least several months. Antimicrobial medication is less labor-intensive than individual vaccination, since antimicrobials can be administered orally. On the other hand, vaccination does not select for antimicrobial resistance and also avoids the risks of antimicrobial residues in the carcasses of slaughter pigs. Although antimicrobials can be often effective against several (respiratory) pathogens and vaccination is mostly focused on the control of *M. hyopneumoniae* infections, other secondary infections causing lung damage are less frequent after vaccination (Maes *et al.*, 1999; Maes *et al.*, 2000). However, even in herds vaccinating against *M. hyopneumoniae*, clinical disease can occur, and antimicrobials may remain necessary (Mateusen *et al.*, 2002). Combined strategies using antimicrobial medication and vaccination are often used under field conditions, especially in herds with a high pressure of *M. hyopneumoniae* infection and/or deficiencies in housing and management. This approach may result in additional clinical and performance benefits (Mateusen *et al.*, 2001; 2002).

Table 7. Comparison of antimicrobial medication *versus* vaccination

Antimicrobial medication	Vaccination
more flexible	long-term strategy
less laborious	more laborious
against different pathogens (<i>e.g.</i> multiple disease challenges)	against one pathogen (combination vaccines possible)
risk for residues	no risk for residues
risk for antibiotic resistance (especially in case of inappropriate or long term use)	no risk for antibiotic resistance
against bacterial infections, not against viruses	vaccines available against limited number of diseases

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Chapter 2. AIMS OF THE STUDY

Aims of the study

AIMS OF THE STUDY

Mycoplasma hyopneumoniae is the primary agent of enzootic pneumonia, a chronic respiratory disease in pigs resulting from mixed respiratory infections with *M. hyopneumoniae* and other bacterial pathogens. *M. hyopneumoniae* infections lead to major economic losses due to the reduced growth, increased mortality and feed conversion, costs for antimicrobials and immunoprophylaxis and increased time to market.

The control of *M. hyopneumoniae* infections can be afforded by three different strategies, namely optimization of housing and management practices, antimicrobial treatment and vaccination. Both, antimicrobial treatment and vaccination have been shown to be able to reduce infections with *M. hyopneumoniae*, but not to eliminate this micro-organism from infected pigs. Despite vaccination, clinical outbreaks of respiratory disease due to *M. hyopneumoniae* infections may still occur. Therefore, better control strategies for this disease are required.

The general aim of this thesis is to obtain better insights regarding different treatment and control strategies against *M. hyopneumoniae* infections.

The specific objectives are:

1. To determine the efficacy of a new florfenicol formulation to treat pigs by means of a single intramuscular injection against experimental *M. hyopneumoniae* infection. Whether this innovative metaphylactic medication established at onset of disease and characterized by a higher concentration of active substance can provide clinical efficacy is unknown. However, it can help to reduce the antimicrobial use in pig herds.

2. To assess the efficacy of a one-shot vaccination applied at either one or three weeks of age in a Belgian farrow-to-finish pig herd with mixed respiratory disease including infections with *M. hyopneumoniae* and viral pathogens late in the fattening period.

3. To compare the efficacy of in-feed medication with chlortetracycline, and tylosin phosphate against a clinical outbreak of respiratory disease in fattening pigs vaccinated against *M. hyopneumoniae*. Whether this chlortetracycline medication established at onset of disease and administered during two consecutives or alternating weeks can provide clinical efficacy is unknown. However, it can help to reduce the antimicrobial use and treatment duration in pig herds.

Chapter 3. **EXPERIMENTAL STUDIES**

Experimental studies

3.1. Efficacy of florfenicol injection in the treatment of *Mycoplasma hyopneumoniae* induced respiratory disease in pigs

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Abstract

This study investigated the efficacy of a single intramuscular injection of florfenicol to treat clinical respiratory disease following experimental *Mycoplasma hyopneumoniae* infection. *M. hyopneumoniae*-free piglets were allocated to three groups namely a treatment (TG) and positive control group (PCG), which were both inoculated endotracheally with a highly virulent isolate of *M. hyopneumoniae*, and a negative control group. At onset of clinical disease, TG received a single injection of a new florfenicol formulation (30 mg/kg). All pigs were euthanized 4 weeks post-infection. Clinical symptoms were significantly reduced in TG in comparison with PCG ($P < 0.05$). Average daily gain, feed conversion ratio, mortality and lung lesions were improved in TG compared to PCG, but the differences were not statistically significant.

Keywords: Pigs; *Mycoplasma hyopneumoniae*; Florfenicol; Treatment

Introduction

Mycoplasma hyopneumoniae plays a primary role in enzootic pneumonia, a chronic respiratory disease that occurs worldwide and causes major economic losses to the pig industry. *M. hyopneumoniae* is also one of the most important agents involved in the porcine respiratory disease complex, together with other bacterial and viral infections. *M. hyopneumoniae* infections lead to reduced performance, increased antimicrobial use and extra costs for control measures such as vaccination (Maes *et al.*, 2008).

Control of *M. hyopneumoniae* infections should be based primarily on optimizing housing and management practices, and on using vaccination. These measures are however not always effective, as even in herds with proper management and housing and applying vaccination, clinical outbreaks of *M. hyopneumoniae* infections may still occur (Maes *et al.*, 2008). Therefore, the use of antimicrobial agents may be warranted and even necessary to treat and/or control *M. hyopneumoniae* infections, and to safeguard the health and welfare of the animals. In such cases, it is important that antimicrobials are used judiciously and that strategic or preventive antimicrobial medication during long periods at risk is minimized (Dewulf *et al.*, 2007). Compared to vaccination, antimicrobial medication has the advantage that it can be implemented more quickly and in a more flexible way, and that antimicrobials may also act against bacterial respiratory pathogens other than *M. hyopneumoniae*.

The most frequently used antimicrobials against *M. hyopneumoniae* are tetracyclines, pleuromutilins, fluoroquinolones and macrolides (Thacker, 2006). Florfenicol is an antibiotic that is widely used in different farm animal species including pigs. It inhibits the microbial protein synthesis by abolishing the activity of the bacterial enzyme peptidyl transferase and is typically classified as a bacteriostatic antibiotic (Lobell *et al.*, 1994). However, bactericidal activity was shown against major swine respiratory pathogens (*A. pleuropneumoniae*, *P. multocida*, *H. parasuis*). Florfenicol is active against many Gram-positive and Gram-negative bacteria, including aerobes, anaerobes, rickettsia, chlamydia and mycoplasmas (Shin *et al.*, 2005).

The aim of this study was to investigate the efficacy of a new florfenicol formulation to treat pigs by means of a single intramuscular injection against experimental *M. hyopneumoniae* infection. The use of a single injection at onset of disease, provided it is effective, could significantly reduce the use of antimicrobials in pig herds in comparison with oral treatments which are usually established for extended periods of time.

Material and methods

Study population, experimental infection and study design

Forty-nine, 3-week-old, cross-bred piglets were purchased from a herd free of *M. hyopneumoniae* and porcine reproductive and respiratory syndrome virus (PRRSV). After weaning (at 3 weeks of age, Day -7), they were transported to the faculty of veterinary medicine (Ghent University). They were randomly allocated in three groups and housed in three different experimental rooms equipped with absolute filters to avoid possible transmission of infection. All pigs received *ad libitum* a commercial, antibiotic-free feed. On day 0 (D0), pigs of the treatment group (TG) ($n = 22$) and the positive control group (PCG) ($n = 22$) were inoculated endotracheally with 7 mL inoculum containing 10^7 color-changing-units/mL of the highly virulent *M. hyopneumoniae* F7.2C strain (Vicca *et al.*, 2003). Pigs of the negative control group (NCG) ($n = 5$) were inoculated with 7 mL of sterile culture medium. For inoculation, the piglets were anesthetized with 0.22 mL/kg of a mixture of xylazine (Xyl-M 2%®, VMD) and zolazepam (Zoletil 100®, Virbac) applied intramuscularly. The treatment was applied when at least 10 per cent of the animals over the two infected groups showed coughing (D14). Animals were injected intramuscularly once with the test florfenicol formulation (Nuflor Swine Once®, MSD Animal Health; florfenicol 450 mg/mL) (TG) or with physiological saline solution (PCG and NCG) at the dose of 1 mL/15 kg bodyweight. All pigs were euthanized on day 28 (D28).

Parameters of comparison

Rectal temperature and severity of coughing were recorded daily for each piglet. The severity of coughing was assessed using a respiratory disease score (RDS) (Halbur *et al.*, 1996). Individual bodyweights and feed intake were measured on D0, D14 and D28 in order to evaluate average daily weight gain (ADG) and feed conversion ratio (FCR). Mortality was recorded during the whole experiment.

After euthanasia, post-mortem parameters were studied. Mycoplasma-like macroscopic lung lesions were quantified using a lung lesion score (Hannan *et al.*, 1982). Two lung tissue samples per lobe (apical, cardiac and diaphragmatic) were collected for histopathological examinations (percentage of tissue occupied by air; severity of peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation) (Morris *et al.*, 1995) and immunofluorescence testing (Kobisch *et al.*, 1978). Bronchoalveolar lavage (BAL) was performed and the fluid recovered was divided into two aliquots. The first aliquot was used

for the detection of *M. hyopneumoniae*-organisms by nested PCR (nPCR) (Stärk *et al.*, 1998) and quantitative PCR (qPCR) (Marois *et al.*, 2010). The second aliquot was used for bacteriological culture to detect the presence of live *M. hyopneumoniae* (Friis, 1975) and other respiratory pathogens (Quinn *et al.*, 1994). Blood samples were collected (D0 and D28) and analysed to detect serum antibodies against *M. hyopneumoniae* and PRRSV. The post-mortem lung lesion score was the primary efficacy criterion. Parameters with continuous variables were analysed using a one-way ANOVA, categorical variables were analysed using chi-square tests.

Results

A graphical representation of the rectal temperatures and RDS is given in Fig. 1 and 2, respectively. Results of clinical, performance, pathological, pathogen recovery and serological parameters in TG and PCG are shown in table 1. All pigs of the infected groups were positive by nPCR in BAL fluid. *M. hyopneumoniae* could not be isolated from the BAL fluid, whereas the routine bacteriological culture led to isolation of other bacterial species, particularly *Bordetella bronchiseptica*, *Haemophilus parasuis* and *Streptococcus suis*. All animals were serologically negative for PRRSV (D28).

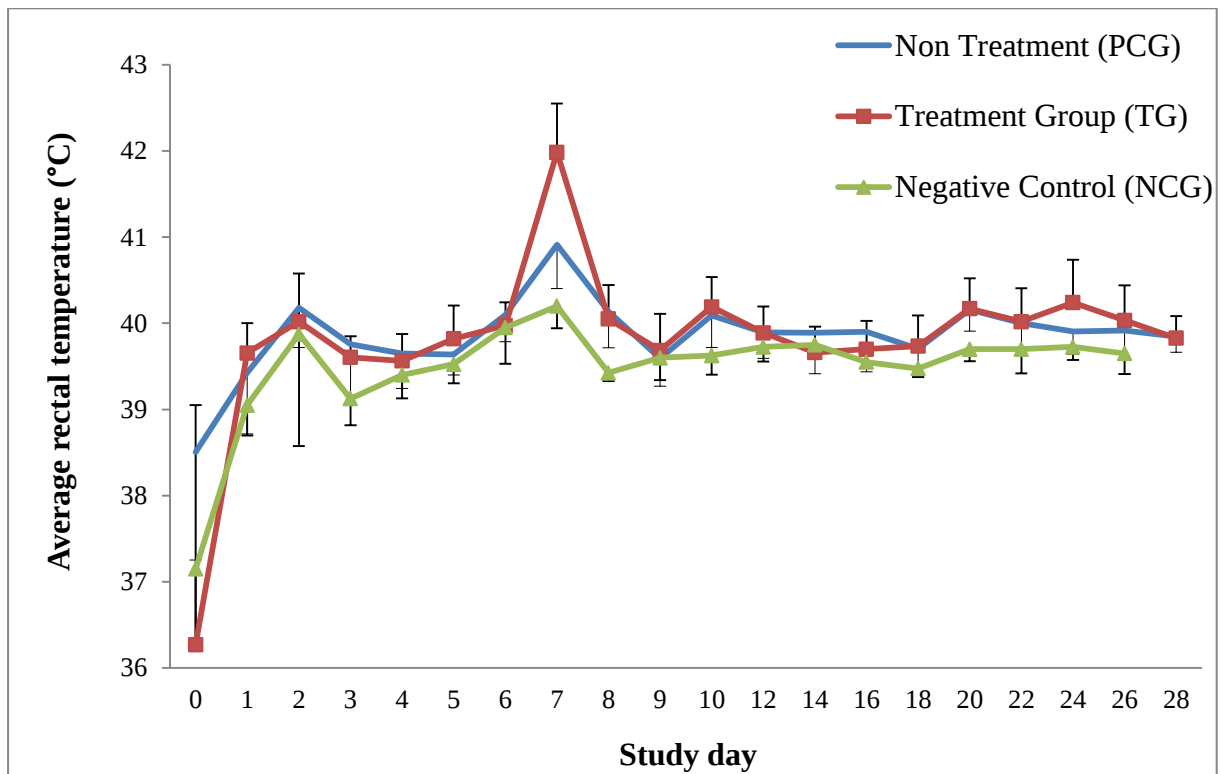


Figure 1. Course of average (+/- SD) Rectal Temperature. Average Rectal Temperature (°C) from day 0 until day 28 in the treatment group (TG = 39.80 ± 0.30 °C), the positive control group (PCG = 39.86 ± 0.24 °C) and the negative control group (NCG = 39.48 ± 0.14 °C). Pigs were challenged endotracheally with a virulent strain of *M. hyopneumoniae* at D0, injected with florfenicol (TG) or physiological saline solution (PCG) at D14, and necropsied at D28.

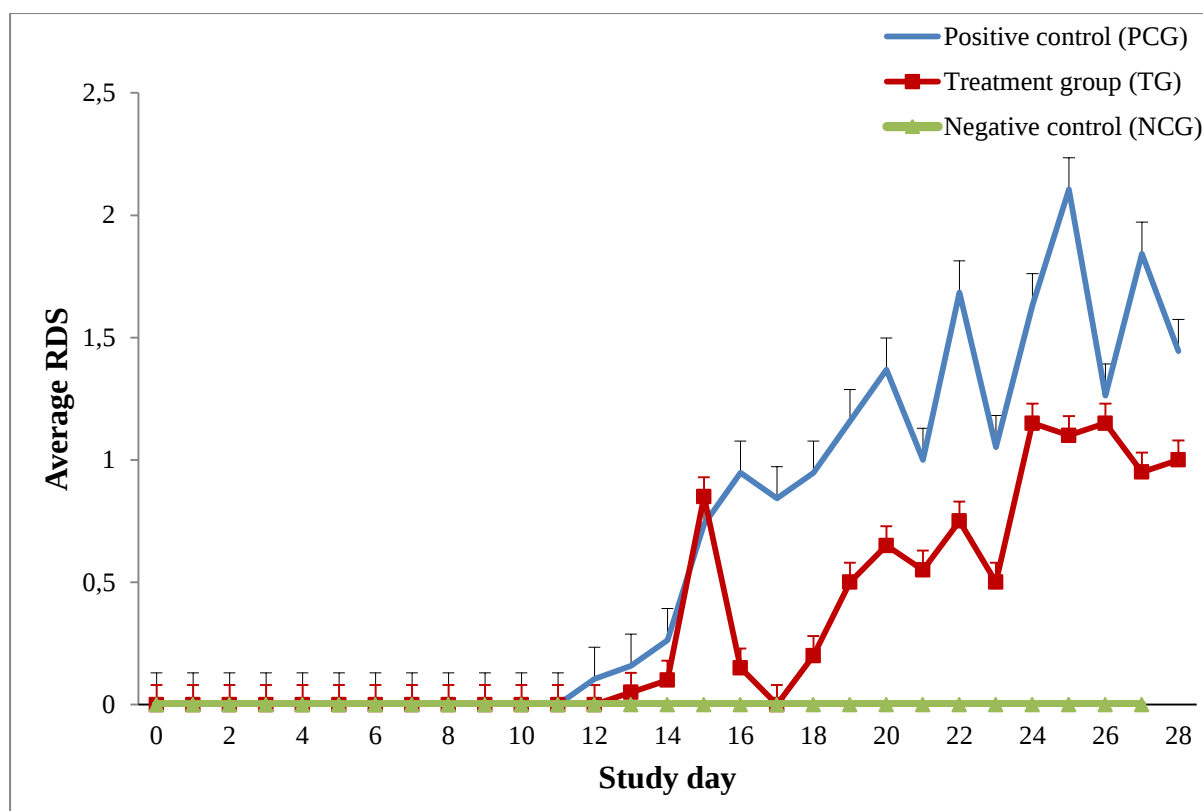


Figure 2. Course of average ($\pm$ SD) respiratory disease score (RDS). Average RDS from day 0 until day 28 in the treatment group (TG), the positive control group (PCG) and the negative control group (NCG). Pigs were challenged endotracheally with a virulent strain of *M. hyopneumoniae* at D0, injected with florfenicol (TG) or physiological saline solution (PCG) at D14 and necropsied at D28.

Table 1. Results (average $\pm$ standard deviation) of the clinical, pathological, performance, infection and serological parameters in the treatment group (TG) and the positive control group (PCG). Pigs were challenged endotracheally with a virulent strain of *M. hyopneumoniae* at D0, injected with florfenicol (TG) or physiological saline solution (PCG) at D14, and necropsied at D28.

	TG <i>n</i> = 20	PCG <i>n</i> = 20	P value
Rectal Temperature (°C)	39.80 $\pm$ 0.30	39.86 $\pm$ 0.24	0.503
RDS D0 to D28	8.69 $\pm$ 7.90	17.16 $\pm$ 12.35	0.024
RDS D14 to D28	8.72 $\pm$ 7.94	17.10 $\pm$ 12.26	0.015
ADG D0 to D28 (g/day)	220 $\pm$ 88	212 $\pm$ 94	0.768
ADG D14 to D28 (g/day)	341 $\pm$ 120	275 $\pm$ 127	0.100
FCR	1.69	2.03	-
Mortality Rate (%)	0.00	10.00	0.232
Macroscopic LLS	4.51 $\pm$ 3.87	5.94 $\pm$ 3.47	0.232
Microscopic LLS	3.85 $\pm$ 0.65	3.72 $\pm$ 0.67	0.540
Percentage Air Analysis	21.94 $\pm$ 6.28	22.20 $\pm$ 5.31	0.892
IF	0.92 $\pm$ 0.73	0.95 $\pm$ 0.65	0.892
qPCR testing: Log copies of <i>M. hyopneumoniae</i> /ml (mean $\pm$ sd)	4.91 $\pm$ 5.96	4.74 $\pm$ 4.92	0.952
% of pigs serologically positive for <i>M. hyopneumoniae</i>	14.3%	26.3%	0.290

Rectal Temperature (average from D0 to D28); RDS (average Respiratory Disease Score; ADG (Average Daily Weight Gain); FCR (Feed Conversion Ratio: no statistical analysis, since FCR has been calculated at pen level, with only one observation); LLS (Lung Lesion Score); Microscopic LLS (peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation); IF (Immunofluorescence); qPCR (quantitative PCR: number of *M. hyopneumoniae*-organisms); per cent of pigs serologically positive for *M. hyopneumoniae* at D28.

Discussion

The present study assessed the efficacy of a florfenicol formulation administered intramuscularly for the treatment of respiratory disease caused by an experimental *M. hyopneumoniae* infection.

According to the RDS, onset of clinical disease took place between 12 and 15 days after endotracheal inoculation and the decision to start the treatment was made on D14. The results showed that the treatment was efficacious only for improving clinical respiratory symptoms. The RDS is indeed an important indicator of the severity of *M. hyopneumoniae* infections (Vicca *et al.*, 2003), and hence to assess the clinical effectiveness of the treatment. The injection of florfenicol significantly decreased the RDS in TG, especially on days 16 and 17. However, this score increased again on day 18, *i.e.* four days post-treatment.

Also, at necropsy (D28 or 29) only a numerical benefit was observed for the macroscopic and the histopathological lung lesions. The percentage of air in the lung tissue and the qPCR results were very similar for the TG and PCG ($P>0.05$). This suggests that the studied parameters may be delayed shortly after treatment, but 14 days later, no significant differences can be found between the groups. Liu *et al.* (2003) reported a long duration of the therapeutic plasma level of florfenicol in pigs experimentally infected with *Actinobacillus pleuropneumoniae*. Our findings suggest that a single injection with this antibiotic only partially controls *M. hyopneumoniae* infection during a period of approximately four days.

All infected pigs were positive by nPCR in the BAL fluid, indicating that the challenge infection was successful and that the treatment did not eliminate *M. hyopneumoniae* from the lungs. The latter confirms previous reports, stating that antimicrobial treatment does not eliminate the pathogen from the lung tissue nor heal existing lesions (Thacker, 2006). Antimicrobial agents may delay infection, but cannot avoid colonization, nor assure total elimination of *M. hyopneumoniae*.

However, *M. hyopneumoniae* could not be isolated from the lungs because of overgrowth of other bacterial species, particularly *Bordetella bronchiseptica*, *Haemophilus parasuis* and *Streptococcus suis* which were isolated in high numbers. All animals were serologically negative for PRRSV (D28).

The performance parameters (ADG, FCR) and mortality were numerically improved in the treated group. More animals would have been needed to obtain statistically significant results, but the tendencies indicate that an economical improvement could be obtained when florfenicol is injected.

The present study showed that the tested florfenicol formulation, when administered intramuscularly as a single dose of 30 mg/kg bodyweight, was effective in reducing the clinical respiratory symptoms in pigs experimentally infected with a highly virulent *M. hyopneumoniae* strain. Despite these findings, optimizing housing and management practices, as well as vaccination should still be the key processes used in the control of *M. hyopneumoniae* infections.

Conflict of interest statement

The study was funded by MSD Animal Health, manufacturer of the antimicrobial used in this study. J. Thiry and E. Thomas are employees of MSD Animal Health.

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3.2. Efficacy of early *Mycoplasma hyopneumoniae* vaccination against mixed respiratory disease in older fattening pigs

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Abstract

The present field study investigated the efficacy of early *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) vaccination in a farrow-to-finish pig herd with respiratory disease late in the fattening period due to combined infections with *M. hyopneumoniae* and viral pathogens. Five-hundred-and-forty piglets were randomly divided into three groups of 180 piglets each: two groups were vaccinated (Stellamune Once®) at either 7 (V1) or 21 days of age (V2), and a third group was left non-vaccinated (NV). The three treatment groups were housed in different pens within the same compartment during the nursery period, and were housed in different but identical compartments during the fattening period. The efficacy was evaluated using performance and pneumonia lesions. The average daily weight gain during the fattening period was 19 (V1) and 18 g/day (V2) higher in both vaccinated groups when compared with the NV group. However, the difference was not statistically significant ($P>0.05$). The prevalence of pneumonia was significantly lower in both vaccinated groups (V1:71.5 and V2:67.1 per cent) when compared with the NV group (80.2 per cent) ($P<0.05$). There were no significant differences between the two vaccination groups. In conclusion, in the present herd with respiratory disease during the second half of the fattening period caused by *M. hyopneumoniae* and viral infections, prevalence of pneumonia lesions were significantly reduced and growth losses numerically (not statistically significant) decreased by both vaccination schedules.

Keywords: *Mycoplasma hyopneumoniae*; pig; early vaccination; one-shot

Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the causative agent of enzootic pneumonia in pigs and is one of the primary agents involved in the porcine respiratory disease complex (PRDC). This disease is distributed worldwide and leads to a significant reduction of economical profit in pig production, mainly due to decreased growth, higher feed conversion ratios and higher costs for treatments (Maes *et al.*, 2008; Thacker and Minion 2012). Control of *M. hyopneumoniae* infections can be accomplished by improving management and housing practices, by the use of antimicrobials and by vaccination (Maes *et al.*, 2008). Vaccination is frequently practiced worldwide. The major advantages of vaccination include a reduction of the losses of average daily gain (ADG; 2-8 per cent), feed conversion ratio (2-5 per cent) and mortality (Maes *et al.*, 2008). Additionally, shorter time to reach slaughter weight, reduced clinical signs and lung lesions and lower treatments costs are observed (Maes *et al.*, 2008).

Different vaccination strategies can be implemented, depending on the type of herd, the production system and management practices, the infection pattern and the preferences of the pig producer. Currently, one-shot vaccination is more often practiced than two-shots vaccination, mainly because it requires less labor, it confers similar results than two-shots vaccination (Roof *et al.*, 2001, 2002; Alexopoulos *et al.*, 2004; Lillie *et al.*, 2004; Greiner *et al.*, 2011), and it can be implemented more easily in routine management practices on the farm. Because *M. hyopneumoniae* infections can take place in piglets already during the first weeks of life (Calsamiglia and Pijoan 2000; Ruiz *et al.*, 2003; Fano *et al.*, 2007; Sibila *et al.*, 2007; Nathues *et al.*, 2010; Villarreal *et al.*, 2010, 2011b; Vranckx *et al.*, 2012a; Fablet *et al.*, 2012a), and because vaccination is most likely effective if active immunity can be established before the exposure to the pathogen, vaccination is commonly applied in suckling piglets. When compared with weaned piglets, suckling piglets are less infected with pathogens, such as porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV-2), which may cause problems after weaning and interfere with the establishment of a protective immune response to *M. hyopneumoniae* vaccination (Maes *et al.*, 2008). Early vaccination has also the advantage that immunity is induced at a young age. This may be important as the onset of infection may vary between herds and also within a herd among successive batches (Sibila *et al.*, 2004b, 2007; Fano *et al.*, 2007; Segalés *et al.*, 2012). Apart from inducing early protection, it is also important that early vaccinated pigs remain

protected until the end of the fattening period, as in most pig herds, the highest infection levels of *M. hyopneumoniae* occur during the grow-finishing period (Sibila *et al.*, 2004c).

Several studies have assessed the efficacy of vaccination at seven days of age against *M. hyopneumoniae* challenge infection under experimental conditions. This early vaccination at seven days of age has been demonstrated effective in reducing lung lesions and/or clinical signs in several challenge infections either at two (Reynolds *et al.*, 2006), four (Reynolds *et al.*, 2009, Villarreal *et al.*, 2011a), six (Meyns *et al.*, 2006), eight (Villarreal *et al.*, 2011a) or nineteen (Kim *et al.*, 2011) weeks post-infection. However, the duration of the effect of one-shot early vaccination under field conditions with mixed respiratory disease is less clear. Mixed respiratory disease is commonly identified as PRDC and results from infections with several primary and secondary respiratory pathogens, such as PRRSV, PCV-2, swine influenza virus (SIV), *M. hyopneumoniae*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Actinobacillus pleuropneumoniae* and *Streptococcus suis* (Opriessnig *et al.*, 2011). As the disease course and lung lesions can be largely influenced by viral respiratory pathogens such as PRRSV (Thacker *et al.*, 1999), PCV-2 (Opriessnig *et al.*, 2004) and swine influenza virus (Thacker *et al.*, 2001), it is important to investigate whether *M. hyopneumoniae* vaccination is also beneficial under such circumstances. The objective of this study was to investigate the efficacy of a one-shot vaccination applied at either one or three weeks of age in a Belgian farrow-to-finish pig herd with mixed respiratory disease late in the fattening period and including infections with *M. hyopneumoniae* and viral pathogens.

Materials and methods

Herd description and study population

The study was conducted between April and October 2011 in a Belgian farrow-to-finish pig herd comprising 1000 commercial hybrid sows (Topigs 20) that operated a four-week batch production system. Semen from Piétrain boars was used for artificial insemination. The sows were vaccinated against swine influenza virus (Gripovac 3®, Merial), Atrophic Rhinitis (Rhinfiffa-T®, Merial), *Escherichia coli* (*E. coli*) (Neocolipor®, Merial), porcine parvovirus and *Erysipelothrix rhusiopathiae*, combined according to the manufacturer's instructions (Parvoruvax®, Merial). The piglets received an iron injection (Uniferon®, Pharmacosmos) (intramuscular, 1 ml/pig) and toltrazuril *per os* (Baycox®, Bayer; 20 mg/kg), both according to the manufacturer's instructions, at three days of age, and they were surgically castrated during the first week of life. Toltrazuril was administered as preventive treatment for coccidiosis. At weaning (three weeks of age), the pigs were moved to a nursery unit where they stayed until 10 weeks of age. Thereafter, they were transferred to a fattening unit, in which they were kept until slaughter weight (approximately 28 weeks of age). Colistin in-feed medication (Promycine 400®, VMD) was prophylactically administered (according to the manufacturer's instructions) after weaning for seven days to prevent problems with post-weaning *E. coli* infections. All pigs received anthelmintic treatment at 10 weeks of age via the feed with flubendazole (Flubenol 5 %®, Elanco Animal Health) for five days.

At weaning, all piglets included in the experiment were allocated into one compartment of the nursery (n=576 pigs). This compartment consisted of 32 pens (18 pigs per pen) with a partially slatted floor and there was mechanical channel ventilation. At 10 weeks of age, all study piglets were moved to six identical compartments of a fattening unit. The three different treatment groups were housed in different compartments (two compartments for each treatment group). Each compartment consisted of eight pens (12 pigs per pen) with a fully slatted floor and there was mechanical door ventilation. The nursery unit and the fattening unit were equipped with an automatic ventilation system controlled by computer. During the entire experiment, water and feed (meal) were supplied *ad libitum*.

Before the start of the study, clinical signs of respiratory disease, namely coughing and sneezing, were evident in nursery piglets from six-to-seven weeks of age onwards. Limited sampling using nasal swabs in 20 two-week-old piglets and broncho-alveolar lavage (BAL) fluids in 20 six-week-old piglets and 10 ten-week-old piglets showed that 0/20, 0/20 and 5/10 piglets, respectively, were positive for *M. hyopneumoniae* by nested polymerase chain

reaction (PCR) (nPCR) (Stärk *et al.*, 1998). Blood samples taken from 20 randomly selected pigs at 19 weeks of age revealed that 75 per cent of the pigs were serologically positive for *M. hyopneumoniae* and 100 per cent for PRRSV using a blocking Enzyme-Linked Immunosorbent Assay (ELISA) (IDEI, *Mycoplasma hyopneumoniae* EIA kit, Oxoid, UK) for *M. hyopneumoniae* and the HerdChek PRRS X3 (IDEXX) for PRRSV. Gross examination of the lungs from fattening pigs (n=826) slaughtered during 12 months before the start of the study indicated an average pneumonia prevalence of 35 per cent.

Experimental design

In total, 540 pigs originating from one batch of sows were selected for this study. Although the original group comprised 250 sows, only the offspring of 60 sows was included in the trial. These 60 sows were selected at random from the group of 250 sows to obtain a representative sample of sows (all parities represented) and piglets. The pigs were individually ear-tagged and randomly allocated to one of the three treatment groups. Within each litter (n=60), an equal number of pigs (n=3) was allocated to each treatment group (blocked randomization). Therefore, in total, each treatment group consisted of 180 pigs. One group was vaccinated intramuscularly at 7 days of age with two ml of a commercial inactivated *M. hyopneumoniae* vaccine (Stellamune Once®, Elanco Animal Health) (designated group V1), a second group was vaccinated intramuscularly at 21 days of age with the same vaccine (designated group V2), a third group was left unvaccinated (designated group NV). Non-vaccinated animals were not injected with a placebo. Upon weaning, piglets were moved to a nursery unit, where they were partly re-grouped according to pen-size, weight and sex. Pigs of the different treatment groups were housed in different pens within the same compartment. The stocking density in the nursery unit was 0.27 m² per pig. Pens were separated by solid pen partitions. As the capacity of the nursery unit (576) was slightly higher than the number of study pigs (540), two pens (36 pigs) were not included in the trial. Twelve of these pigs had been vaccinated at one week, twelve at three weeks, and twelve had not been vaccinated.

The pigs were moved to a fattening unit at approximately 70 days of age. At that time, they were partly re-grouped according to pen size and weight. Barrow and gilts were housed in different pens, and pigs of different treatment groups were housed in different compartments (two compartments for each treatment group). The 36 extra pigs were divided in three pens, one in each treatment group. They were not included in the study as the approval for the ethical committee before the study included 540 pigs. The stocking density

in the fattening unit was 0.75 m² per pig. Fattening pigs of neighbouring pens could have direct nose-to-nose contact. All other factors that could possibly influence the outcome parameters *e.g.* environment and management factors, sex ratio, and feed composition were the same for the three groups. The person responsible for assessing the efficacy parameters was unaware of the allocation of the groups. All animals were housed and reared in accordance with EU animal welfare regulations and in accordance with EU experimental animal legislation (Directive 86/609/EEC). The study was approved by the ethical committee for animal experiments of the Faculty of Veterinary Medicine, Ghent University (EC2011/068).

Parameters of comparison

Performance parameters

Pigs were individually weighed at one week of age (7 days), at weaning (21 days), at the end of the nursery period (74 days) and prior to slaughter (200 days) to determine the average daily gain (ADG). The ADG (g/pig/day) was computed for the different production stages (lactation, nursery and fattening period) as the difference between starting and final weights divided by the number of days during that period.

Clinical and pathological parameters

All pigs that died during the study were necropsied to assess the possible cause of death. Where appropriate, necropsied pigs were processed for further laboratory examinations. When the dead animals were suspected to be infected with *M. hyopneumoniae*, nPCR testing (Stärk *et al.*, 1998) was performed on the lungs. DNA was extracted using the DNA easy kit (Quiagen, Blood and tissue kit, Belgium).

When individual signs of respiratory disease (coughing, sneezing, dyspnea, tachypnea) were observed by the farmer and confirmed by the veterinarian, individual treatment against respiratory disease was allowed. Concomitant antimicrobial treatment of study pigs (parenterally or orally) was only allowed with antimicrobials ineffective against *M. hyopneumoniae*, such as β -lactam antibiotics (penicillins and cephalosporins). For parenteral treatment, ceftiofur (Excenel RTU®, Pfizer Animal Health) or amoxicillin (Duphamox Long Acting®, Pfizer Animal Health) could be used, for group treatment, amoxicillin applied via the drinking water (Amoxicilline 70 %®, Kela Laboratoria) could be used. The total number

of treatment days against respiratory disease (based on individual or group treatment) was recorded in the different treatment groups.

At slaughter (201-214 days of age), the prevalence of *Mycoplasma*-like pneumonia lesions and adhesive pleurisy and the extent of macroscopically visible pneumonia were recorded by the first author in a blinded manner. *Mycoplasma*-like pneumonia lesions were defined as red to purplish consolidated areas on the cranio-ventral portions of the lungs in a lobular pattern. Adhesive pleurisy was defined as fibrotic adherence between the visceral and parietal membranes of the pleural sac. Due to practical reasons, only the visceral membrane was evaluated for the detection of pleurisy. The extent of macroscopically visible pneumonia was quantified based on a score described by Morrison *et al.* (1985). Briefly, this lung lesion score expresses the total area (percentage) of the lung tissue affected by pneumonia. Each lobe represents a percentage of the total lung surface area: apical (10 per cent), cardiac (7 per cent), accessory (6 per cent) and diaphragmatic (30 per cent). An average score was calculated for each group based on the lung lesion score of the individual animals.

Detection of *M. hyopneumoniae* by nested PCR (nPCR)

Nasal swabs, tracheo-bronchial swabs (Marois *et al.*, 2007; Fablet *et al.*, 2010) and broncho-alveolar lavage (BAL) fluids (Villarreal *et al.*, 2011b) were collected from approximately 30 randomly selected animals per group that were serially sampled at different ages (Table 1). Nasal swabs (Copan®, Italy) were collected at weaning (21 days), halfway through the nursery period (49 days), at the end of the nursery period (74 days), halfway through the fattening period (136 days) and prior to slaughter (200 days). Tracheo-bronchial swabs (Euromedis®, Neuilly-sous-Clermont, France) were collected at the same time points as the ones for nasal swabs, but not at 200 days of age. BAL fluids were collected at 49, 74 and 136 days of age. DNA was extracted with the DNA easy kit (Quiagen, Blood and tissue kit, Belgium) and a nPCR was performed (Stärk *et al.*, 1998).

Serological examinations

Blood samples were collected from the same 30 animals per group (Table 1) at 7, 21, 74, 136, and 200 days of age. Serological examination to detect antibodies to *M. hyopneumoniae* was carried out using a blocking ELISA (IDEI, *Mycoplasma hyopneumoniae* EIA kit, Oxoid, UK). Sera with optical density (OD) values <50 per cent of the OD_{buffer-control} were considered as positive. Sera with OD values ≥50 per cent of the OD_{buffer-control} were considered as negative. Fifteen blood samples were randomly selected from the 30 blood

samples collected at 74, 136, and 200 days of age and additionally tested for antibodies to PRRSV (HerdChek PRRS X3, IDEXX), PCV-2 (Ingezim Circovirus IgG/IgM, Ingenasa), subtypes H1N1, H1N2, and H3N2 of swine influenza virus (SIV) (standard haemagglutination-inhibition test), and serotype 2 (Swinecheck APP 2, Biovet) and serotypes 1, 9 or 11 (Swinecheck APP 1,9,11, Biovet) of *Actinobacillus pleuropneumoniae*.

Carcase quality

At slaughter, carcase quality, including the percentage of meat, back fat thickness and muscle thickness of the *Longissimus dorsi*, was automatically recorded by the abattoir services.

Statistical analysis

The number of animals in each treatment group (180) allowed to assess a difference of 20 g (sd=70) in ADG with 95 per cent certainty and 80 per cent statistical power (Win Episcope 2.0, CLIVE, Edinburgh, UK). The pig was considered as statistical unit. To correct for the effects of hierarchical data, pen and compartment were included as random effects. Analysis of variance (ANOVA) models were used to analyse the continuous variables (weight, number of treatment days against respiratory disease, carcase quality parameters). Some other continuous variables (ADG and lung lesion scores) did not fulfill the criteria of normality and homogeneity of variances, and they were analysed using a non-parametric Kruskal-Wallis ANOVA. Mortality and prevalence of pneumonia and pleurisy were analysed using logistic regression analysis. Differences were considered as statistically significant when P-values were lower than 0.05 (two-sided test). Statistical analyses were performed using the software package SPSS version 20.0 (SPSS Institute Inc., Illinois, USA). Results are presented as mean $\pm$ sd unless stated otherwise.

Table 1. Sampling scheme for nasal (NS) and tracheo-bronchial swabs (TBS), broncho-alveolar lavage (BAL) fluids and blood samples. Thirty pigs per group were randomly selected. NS, TBS and BAL fluid samples were analysed using nested PCR to detect the presence of *M. hyopneumoniae*, blood samples were analysed using ELISA to detect antibodies to *M. hyopneumoniae*.

Age (d) of sampling*	NS	TBS	BAL fluids	Blood
7	no	no	no	yes
21	yes	yes	no	yes
49	yes	yes	yes	no
74	yes	yes	yes	yes
136	yes	yes	yes	yes
200	yes	no	no	yes

*At 7 (one week), 21 (at weaning), 49 (halfway nursery period), 74 (end of nursery period), 136 (halfway fattening period) and 200 days (prior to slaughter).

Results

Performance parameters

The weights of the pigs at 7, 21, 74, and 200 days of age are presented in Table 2. On average, pigs of the V1 and V2 groups gained 2.3 kg (group V2) to 2.4 kg (group V1) more than pigs in the NV group between 7 and 200 days of age. The ADG during the lactation period (7 to 21 days), the nursery period (21 to 74 days), the fattening period (74 to 200 days), and during the entire study period (7 to 200 days) are presented in Table 3. Over the entire fattening period, the ADG in the V1 and V2 group was 19 and 18 g/pig/day, respectively, higher than in the NV group. These differences were not statistically significant ($P>0.05$).

Clinical and pathological parameters

None of the pigs died between the first and second vaccination. In total, 15 pigs (2.8 per cent) died from 21 days of age until slaughter. Four of them died following either sampling or weighing (1 in the V1 group, 3 in the NV group). The other dead animals occurred as follows in the three treatments groups: V1 ($n=4$; 2.2 per cent), V2 ($n=1$; 0.6 per cent), and NV ($n=6$; 3.3 per cent) ($P>0.05$). Pneumonia was the major post-mortem finding in five out of 15 necropsied pigs (1 in the V2 group, 4 in the NV group). *Mycoplasma hyopneumoniae* was not detected by nPCR in any of these five lungs.

Sporadic non-productive coughing was observed at approximately 151 days of age. Pigs showing clinical respiratory disease signs were treated parenterally with long-acting amoxicillin or ceftiofur (V1=13; V2=8; NV=9). At 161 days of age, respiratory disease was observed in many pigs of the three groups. The three groups were more or less similarly affected. Therefore, all pigs of the study were treated for five days with amoxicillin via the drinking water. The total numbers of days ($\pm$ sd) of additional antibiotic treatment were 5.06 ± 0.28 (V1), 5.02 ± 0.13 (V2) and 5.03 ± 0.26 (NV) ($P>0.05$).

At slaughter, the prevalence of *Mycoplasma*-like pneumonia lesions and pleurisy and the extent of macroscopically visible pneumonia were recorded in 505 pigs (V1: 165; V2: 173; NV: 167). Because of practical reasons (pigs with lost ear tags and/or lungs that did not reach the examination stand at the slaughterhouse), a few plucks could not be scored in the slaughterhouse (10, 6 and 4 in V1, V2 and NV, respectively).

The percentage of pigs with pneumonia was significantly lower in both vaccinated groups V1 (118/165 pigs; 71.5 per cent) and V2 (116/173 pigs; 67.1 per cent) when

compared with the NV group (134/167 pigs; 80.2 per cent) pigs ($P<0.05$). The prevalence of pleurisy was also lower in both vaccinated groups (V1, 7.6 per cent; V2, 11.6 per cent) when compared with NV group (12.3 per cent), however it was not statistically significant ($P>0.05$). The average pneumonia scores in the V1, V2, and NV group were 13.0, 9.8, and 14.4, respectively ($P<0.05$) (Table 2). In accordance with this score, 51 per cent of the pigs presented mild pneumonia lesions characterized by an extent lower than 10 per cent of the overall lung surface, which is indicative of a recent (late in fattening period) and mild respiratory disease.

Table 2. Average ($\pm$ sd) bodyweight at 7, 21, 74, and 200 days (d) of age, and carcase quality (% meat, back fat thickness and muscle thickness), prevalence and extent of pneumonia and prevalence of pleurisy at slaughter in pigs vaccinated at 7 days of age (V1), 21 days of age (V2) and in pigs not vaccinated (NV) against *Mycoplasma hyopneumoniae*.

Parameters	Age (d) [†]	V1	V2	NV
Number of pigs at the beginning		<i>n</i> =180	<i>n</i> =180	<i>n</i> =180
Average bodyweight (kg)	7	2.51 $\pm$ 0.56	2.57 $\pm$ 0.60	2.53 $\pm$ 0.66
	21	5.42 $\pm$ 1.05	5.58 $\pm$ 1.04	5.54 $\pm$ 1.11
	74	22.95 $\pm$ 4.24	23.04 $\pm$ 3.77	23.20 $\pm$ 4.82
	200	119.79 $\pm$ 14.13	119.67 $\pm$ 13.10	117.42 $\pm$ 14.86
Mortality (%)		4/180 (2.2)	1/180 (0.6)	6/180 (3.3)
Number of pigs at slaughter		<i>n</i> =165*	<i>n</i> =173	<i>n</i> =167
Prevalence of pneumonia (%)	201-214	71.5 ^A	67.1 ^A	80.2 ^B
Prevalence of pleurisy (%)	201-214	7.6	11.6	12.3
Extent of pneumonia	201-214	12.96 $\pm$ 14.36 ^{AB}	9.76 $\pm$ 10.61 ^A	14.38 $\pm$ 14.43 ^B
% Meat	201-214	59.99 $\pm$ 4.05	61.47 $\pm$ 3.56	60.12 $\pm$ 3.85
Back fat thickness (mm)	201-214	17.29 $\pm$ 3.67	14.93 $\pm$ 2.82	15.89 $\pm$ 3.58
Muscle thickness (mm)	201-214	66.61 $\pm$ 7.43	65.46 $\pm$ 5.94	64.65 $\pm$ 6.80

^{A,B} Values with different superscripts within a row are significantly different ($P < 0.05$).

*The number of pigs that were investigated at slaughter in groups V1, V2 and NV were 165, 173 and 167, respectively.

[†] 201-214 days = slaughter age

Table 3. Average ($\pm$ sd) daily weight gain (ADG) during the lactation period (7-21 days), the nursery period (21-74 days), the fattening period (74-200 days), and during the entire study period (7-200 days) in pigs vaccinated at 7 days of age (V1), at 21 days of age (V2) and not vaccinated (NV) against *Mycoplasma hyopneumoniae*. Differences between groups were not statistically significant ($P>0.05$).

Age range (days)	ADG ($\pm$ sd) (g/pig/day)		
	V1	V2	NV
7-21	209 $\pm$ 46	215 $\pm$ 47	216 $\pm$ 44
21-74	334 $\pm$ 62	329 $\pm$ 59	337 $\pm$ 67
74-200	771 $\pm$ 89	770 $\pm$ 88	752 $\pm$ 100
7-200	610 $\pm$ 67	608 $\pm$ 66	598 $\pm$ 73

Detection of *M. hyopneumoniae* by nested PCR (nPCR)

The numbers of nPCR-positive nasal swabs, tracheo-bronchial swabs and BAL fluids at 21, 49, 74, 136, and 200 days of age are presented in Table 4. All samples tested at 21 and 49 days of age were negative for *M. hyopneumoniae*. At 74 days of age, the percentage of *M. hyopneumoniae*-positive tracheo-bronchial swabs and BAL fluids in the non-vaccinated (NV) group was 7.7 per cent (2/26 pigs) and 4.0 per cent (1/25 pigs), respectively, whereas all samples collected at that age in the vaccinated groups remained negative. At 136 days of age, the percentage of *M. hyopneumoniae*-positive BAL fluids was 24.1 per cent (7/29 pigs), 20.0 per cent (5/25 pigs), and 30.4 per cent (7/23 pigs) in the V1, V2, and NV group, respectively. At 200 days of age, the percentage of *M. hyopneumoniae*-positive nasal swabs was 14.3 per cent (4/28 pigs), 8.7 per cent (2/23 pigs) and 23.1 per cent (6/26 pigs) in the V1, V2, and NV group, respectively.

Serological examinations

The number of pigs seropositive for *M. hyopneumoniae* at 7, 21, 74, 136, and 200 days of age is presented in Table 5. Briefly, the percentage of seropositive pigs remained low during lactation (7 days: 13 per cent; 21 days: 14 per cent), decreased during the nursery period (74 days: 2 per cent) and increased during the fattening period (136 days: 5 per cent; 200 days: 21 per cent).

The number of pigs seropositive for PRRS(V), PCV-2 (IgG and IgM), subtypes H1N1, H1N2 and H3N2 of SIV, serotypes 1, 9 or 11 and serotype 2 of *A. pleuropneumoniae* at 74, 136, and 200 days of age is presented in Table 6.

Carcase quality

The results of the carcase quality are included in Table 2. There were no significant differences between the different groups ($P>0.05$).

Table 4. Results of nested polymerase chain reaction (nPCR) assays on nasal swabs (NS), tracheo-bronchial swabs (TBS) and broncho-alveolar lavage fluids (BALF) at 21, 49, 74, 136, and 200 days (d) of age in pigs vaccinated at 7 days of age (V1), vaccinated at 21 days of age (V2) and not vaccinated (NV) against *Mycoplasma hyopneumoniae*.

Age (d) of pigs at sampling	Number (%) of nPCR-positive pigs/total number of pigs sampled								
	V1			V2			NV		
	NS	TBS	BALF	NS	TBS	BALF	NS	TBS	BALF
21	0/30 (0.0%)	0/30 (0.0%)	NA*	0/30 (0.0%)	0/30 (0.0%)	NA	0/30 (0.0%)	0/30 (0.0%)	NA
49	0/30 (0.0%)	0/30 (0.0%)	0/30 (0.0%)	0/30 (0.0%)	0/30 (0.0%)	0/30 (0.0%)	0/30 (0.0%)	0/30 (0.0%)	0/30 (0.0%)
74	0/29 (0.0%)	0/29 (0.0%)	0/29 (0.0%)	0/28 (0.0%)	0/28 (0.0%)	0/28 (0.0%)	0/26 (0.0%)	2/26 (7.7%)	1/25 (4.0%)
136	0/29 (0.0%)	4/29 (13.8%)	7/29 (24.1%)	0/26 (0.0%)	0/26 (0.0%)	5/25 (20.0%)	4/25 (16.0%)	7/25 (28.0%)	7/23 (30.4%)
200	4/28 (14.3%)	NA	NA	2/23 (8.7%)	NA	NA	6/26 (23.1%)	NA	NA

*NA: not applicable

Table 5. Number (%) of pigs with serum antibodies to *Mycoplasma hyopneumoniae* at 7, 21, 74, 136, and 200 days (d) of age in pigs vaccinated at 7 days of age (V1), vaccinated at 21 days of age (V2) and not vaccinated (NV) against *Mycoplasma hyopneumoniae*.

Age (d) of pigs at sampling	Number (%) of seropositive pigs for <i>M. hyopneumoniae</i> /total number of pigs sampled		
	V1	V2	NV
7	5/30 (16.7%)	3/30 (10.0%)	4/30 (13.3%)
21	3/29 (10.3%)	5/29 (17.2%)	4/30 (13.3%)
74	2/29 (6.9%)	0/27 (0.0%)	0/30 (0.0%)
136	0/27 (0.0%)	2/24 (8.3%)	2/27 (7.4%)
200	8/26 (30.8%)	2/23 (8.7%)	6/27 (22.2%)

Table 6. Number of pigs with serum antibodies to PRRSV, PCV-2 (IgM and IgG), SIV (subtypes H1N1, H1N2 and H3N2) and *A. pleuropneumoniae* (serotypes 1, 9, 11 and 2) at 74, 136 and 200 days (d) of age in pigs vaccinated at 7 days of age (V1), vaccinated at 21 days of age (V2) and not vaccinated (NV) against *Mycoplasma hyopneumoniae*.

	Number of seropositive pigs/total number of pigs sampled								
	V1			V2			NV		
	74 d	136 d	200 d	74 d	136 d	200 d	74 d	136 d	200 d
PRRSV	1/15	13/13	12/12	0/14	15/15	11/11	0/14	13/14	14/14
PCV-2 IgM	0/15	3/13	1/12	0/14	8/15	0/11	0/14	10/14	0/14
PCV-2 IgG	0/15	1/13	12/12	0/14	8/15	10/11	0/14	7/14	14/14
Influenza H1N1	15/15	1/13	11/12	14/14	2/14*	11/11	14/14	2/12*	14/14
Influenza H1N2	15/15	2/13	5/12	14/14	1/14*	8/11	14/14	2/12*	6/14
Influenza H3N2	12/15	0/13	0/12	6/14	0/14*	1/11	8/14	0/12*	0/14
<i>A. pleuropneumoniae</i> Serotype 1, 9, 11	0/15	0/13	0/12	0/14	0/15	0/11	0/14	0/14	0/14
<i>A. pleuropneumoniae</i> Serotype 2	0/15	0/13	0/12	0/14	0/15	7/11	0/14	0/14	2/14

*At 136 days of age, 15 and 14 pigs were sampled in V2 and NV groups, but three blood samples (one from V2; two from NV group) were collected in a limited amount and analysis for swine influenza viruses could not be performed, therefore results are shown over the total number of samples analyzed (V2: n=14; NV: n=12).

Discussion

The present study demonstrated that a single vaccination against *M. hyopneumoniae* applied at either 7 or 21 days of age reduced the prevalence and extent of pneumonia lesions in a pig herd with clinical respiratory disease during the second half of the fattening period. Numerical, but not statistically significant improvements of performance and reduction of the prevalence of pleurisy lesions were observed.

The clinical respiratory disease signs occurred first in a few pigs at approximately 150 days of age, and one-to-two weeks later, more pronounced clinical signs were observed in many pigs. Therefore, initially only individual treatments were applied, whereas in the second stage, a group treatment with antimicrobials was initiated. Based on the diagnostic testing, it is clear that infections with many different pathogens had occurred from approximately halfway through the fattening period and onwards. At slaughter age, almost all pigs were seropositive for PRRSV, PCV-2 and H1N1, and approximately half of them for H1N2. Infections with *M. hyopneumoniae* were also involved, but the precise role of *M. hyopneumoniae* infections in the present outbreak is difficult to assess. At day 136, 20-30 per cent of the pigs were positive based on nPCR testing of BAL fluid, but the serological prevalence at slaughter age was rather low namely approximately 10-30 per cent. Andreasen *et al.* (2001) also found a low seroprevalence of *M. hyopneumoniae* close to slaughter in pigs with high percentage of pneumonia at slaughter. This may be due to the long and variable time (3-to-11 weeks) needed for seroconversion after infection with this pathogen (Morris *et al.*, 1995). *A. pleuropneumoniae* had likely not played a major role as most of the animals remained seronegative at slaughter age, the mortality remained rather low and no typical lesions were found in the dead animals at postmortem examination. The severity of the clinical signs warranted an antimicrobial treatment, but in general, the signs were not very severe and a short group treatment of five days was sufficient. However, there was a very high percentage of pigs with pneumonia lesions (80 per cent in the NV group) at slaughter age. This high percentage can likely be explained by the fact that multiple viral infections and *M. hyopneumoniae* were involved, and that the infections mainly took place during the second half the fattening period, allowing insufficient time for the lesions to be healed at slaughter age (Blanchard *et al.*, 1992). Pneumonia lesions at slaughter are not pathognomonic for infections with *M. hyopneumoniae*. Apart from *M. hyopneumoniae*, it is well known that infections with viruses and in particular swine influenza virus may cause similar pneumonia lesions and may be involved in PRDC (Thacker *et al.*, 2001; Sibila *et al.*, 2009).

The respiratory problems in the present batch were different from those in the previous batches in this herd. They occurred later in the present batch, *M. hyopneumoniae* infections were less prevalent and mainly occurred from halfway the fattening period and onwards (serology and PCR on swabs and BAL fluid), and there were complicating viral infections (serology). In previous batches, respiratory problems mainly occurred in the nursery and were clearly related to *M. hyopneumoniae* infections. This confirms that the infection pattern of *M. hyopneumoniae* is not constant over time within the same herd, and that important variations may occur between successive batches (Fano *et al.*, 2007; Sibila *et al.*, 2007). Previous studies showed a seasonal variation of *M. hyopneumoniae* infections, *i.e.* higher prevalence and severity of lung lesions (Straw *et al.*, 1986), higher seroprevalence to *M. hyopneumoniae* (Maes *et al.*, 2000), and higher probability of being *M. hyopneumoniae*-PCR positive (Segalés *et al.*, 2012) during winter period. The absence of a constant infection pattern, *i.e.* infection in younger or older pigs depending on the batch, is one of the major reasons why vaccination of piglets during the first weeks of life is commonly practiced worldwide. Using this strategy, piglets are immunized before infection, irrespective of whether infection takes place in the nursery, growing or fattening unit.

Both vaccination schedules (V1 and V2) led to a numerical, but not statistically significant, increase of the ADG during the fattening period of 19 and 18 g/pig/day, respectively, when compared with the NV pigs. The fact that the improvement was not statistically significant may be due to the high standard variations (88-100 g/day), the fact that the respiratory problems occurred only late in the fattening period, and that different viral infections were involved in the outbreak. Similar improvements in ADG following *M. hyopneumoniae* vaccination were obtained in a meta-analysis of 28 published field trials (Jensen *et al.*, 2002). In that study, the ADG in pigs vaccinated against *M. hyopneumoniae* was on average 21 g higher when compared with non-vaccinated pigs. An ADG improvement of 20 g is associated with an important economic profit (Maes *et al.*, 2003). Additionally, Pagot *et al.* (2007) described a negative association between growth during fattening period and prevalence of pneumonia at slaughter. Our study results corroborate with these observations: in a population with high prevalence of pneumonia (67 to 80 per cent), vaccination results in an increase of 2.5 per cent of ADG, when compared with non-vaccinated pigs.

Vaccination also significantly reduced the percentage of slaughter pigs with pneumonia lesions, which is in agreement with previous studies using challenge infections more than five

months after vaccination (Reynolds *et al.*, 2009). Our data showed a low prevalence of pleurisy (7-12 per cent) in the slaughter pigs of this herd, when compared with prevalence figures (*i.e.* 15 to 60 per cent) obtained in several epidemiological studies carried out recently in Europe (Fraile *et al.*, 2010; Meyns *et al.*, 2011; Fablet *et al.*, 2012b; Merialdi *et al.*, 2012). This low prevalence of pleurisy may indicate a low impact on respiratory disease in this study.

In addition, the percentage of pigs that tested positive with PCR was lower and positive pigs were detected at a later stage in the vaccinated groups than in the control group, illustrating that vaccination had decreased the infection level of *M. hyopneumoniae* in this herd during the experiment. By using qPCR, Vranckx *et al.* (2012b) recently showed that vaccination also significantly reduced the number of *M. hyopneumoniae* organisms in the lungs of experimentally infected pigs. The fact that *M. hyopneumoniae* DNA could still be detected by PCR confirms previous results from previous experimental and field transmission studies with different *M. hyopneumoniae* vaccines (Meyns *et al.*, 2006; Villarreal *et al.*, 2011b). These studies showed that vaccination does not prevent infection, and suggested that vaccination alone is not able to eliminate *M. hyopneumoniae* from infected pig herds.

More *M. hyopneumoniae*-positive pigs were found using nPCR in both tracheo-bronchial swabs and BAL fluids than in nasal swabs. This confirms previous reports (Kurth *et al.*, 2002; Sibila *et al.*, 2004a, 2007; Marois *et al.*, 2007; Fablet *et al.*, 2010) and is consistent with the fact that not the nose, but the trachea and bronchi are the main multiplication sites of *M. hyopneumoniae* (Blanchard *et al.*, 1992). In total, 90 pigs were restrained five times for collecting blood and BAL fluid, tracheo-bronchial and nasal swabs samples. These sampling procedures likely caused stress to the animals and may have increased their susceptibility to respiratory disease and affected growth. The number of sampled pigs was the same in three groups. To keep a sufficient number of animals per group in the study, the sampled pigs were also included for the data analysis.

Assuming that the detected antibodies at 7 and 21 days of age in all three groups are maternally-derived antibodies, the present study further confirms that vaccination of piglets against *M. hyopneumoniae* either in the first or third week of age in the presence of maternally-derived serum antibodies is efficacious to control *M. hyopneumoniae* infections (Martelli *et al.*, 2006; Ritzmann *et al.*, 2006; Reynolds *et al.*, 2009). The low percentages (<10 per cent) of pigs seropositive for *M. hyopneumoniae* at 74 and 136 days of age in both vaccinated groups further confirms that one-shot vaccination does not lead to significant

increases in serum antibodies, and that serum antibody concentrations are not correlated with protection against *M. hyopneumoniae* (Djordjevic *et al.*, 1997; Thacker *et al.*, 1998). It also implies that testing for the presence of serum antibodies is not reliable to evaluate whether pigs have been vaccinated properly in herds endemically infected with *M. hyopneumoniae*.

In conclusion, the present study demonstrated that vaccination against *M. hyopneumoniae* at 7 or 21 days of age significantly reduced pneumonia lesions in a herd with clinical respiratory problems during the second half of the fattening period due to combined *M. hyopneumoniae* and viral infections. A numerical, but not statistically significant reduction of growth losses was also observed.

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3.3. Efficacy of in-feed medication with chlortetracycline in a farrow-to-finish herd against a clinical outbreak of respiratory disease in fattening pigs

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Abstract

The efficacy of chlortetracycline in-feed medication to treat pigs with clinical respiratory disease was investigated in a farrow-to-finish pig herd infected with *Mycoplasma hyopneumoniae* and with clinical respiratory disease in growing pigs. In total, 533 pigs were included. The animals were vaccinated against *M. hyopneumoniae* and porcine circovirus type 2 at weaning. At onset of clinical respiratory disease, they were randomly allocated to one of the following treatment groups: chlortetracycline 1 (CTC1) (two consecutive weeks, 500 ppm), chlortetracycline 2 (CTC2) (two non-consecutive weeks, with a non-medicated week-interval in between, 500 ppm) or tylosin (T) (three consecutive weeks, 100 ppm). Performance (daily weight gain, feed conversion ratio), pneumonia lesions at slaughter and clinical parameters (respiratory disease score) were assessed. Only numeric differences in favour of the CTC2 group were obtained for the performance and the clinical parameters. The prevalence of pneumonia lesions was 20.5^{AB}, 13.1^A and 23.0^B per cent ($P < 0.05$) for the CTC1, CTC2 and T groups, respectively. The study demonstrated that chlortetracycline, when administered at onset of clinical respiratory disease via the feed at a dose of 500 ppm during two alternative weeks, was able to decrease the prevalence of pneumonia lesions, and numerically reduce performance losses and clinical signs.

Keywords: Pig; *Mycoplasma hyopneumoniae*; Treatment; Chlortetracycline

Introduction

The porcine respiratory disease complex (PRDC) causes major economic losses to the swine industry. One of the main pathogens of PRDC is *Mycoplasma hyopneumoniae* (Dee, 1996), which together with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV-2), are considered to play an important role in this complex (Opriessnig *et al.*, 2004; Thacker, 2006; Sibila *et al.*, 2009). Moreover, *M. hyopneumoniae* is also the primary etiological agent of enzootic pneumonia, a chronic respiratory disease in swine. The organism attaches to cilia and colonizes the mucosal surface of the ciliated epithelium of the trachea, bronchi and bronchioles. This adhesion to the cilia on the epithelial surface provokes a disruption in the mucosal clearance system and predisposes to concurrent infections with other respiratory pathogens (Maes *et al.*, 2008), such *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, *Pasteurella multocida* and *Streptococcus suis* (Opriessnig *et al.*, 2011). Additionally, the organism modulates the immune system of the respiratory tract (Thacker *et al.*, 2006). Mycoplasmal pneumonia is characterised by chronic non-productive coughing, reduced growth rate and higher feed conversion ratio in grow-finishing pigs. This decreased performance of the pigs, as well as the increased use of medication to treat and control respiratory disease, imply additional economic losses to pig producers (Maes *et al.*, 2008).

Control of *M. hyopneumoniae* infections can be achieved by different strategies, such as improvements in management and housing conditions, vaccination and antimicrobial treatment. Vaccination has been demonstrated in previous field studies to significantly reduce performance losses and the severity of clinical signs and lung lesions (Maes *et al.*, 1998, 1999, Jensen *et al.*, 2002). However, vaccination is not able to prevent colonization of the respiratory tract with *M. hyopneumoniae*, to significantly reduce the transmission of *M. hyopneumoniae*, nor to eliminate *M. hyopneumoniae* from infected herds (Meyns *et al.*, 2006, Villarreal *et al.*, 2011). Consequently, respiratory disease problems caused by *M. hyopneumoniae* infections are only partially alleviated by vaccination, and in some herds, clinical problems may still be present. In that case, pigs need to be medicated with antimicrobials active against *M. hyopneumoniae* such as tetracyclines, macrolides, pleuromutilins, lincosamides, fluoroquinolones, aminocyclitols, aminoglycosides and florfenicol (Vicca, 2005).

The present study compared the efficacy of in-feed medication with chlortetracycline and tylosin phosphate against a clinical outbreak of respiratory disease in fattening pigs.

Chlortetracycline is a broad spectrum bacteriostatic antimicrobial. It inhibits bacterial protein synthesis, by irreversible binding to receptors of the 30S bacterial ribosome (Prescott *et al.*, 2000). It is active against many Gram-positive and Gram-negative bacteria, chlamydia, rickettsia, mycoplasmas and some protozoa. Tetracyclines, as amphoteric molecules, diffuse readily through biological barriers. Oral bioavailability of tetracyclines is very low when administered as in-water or in-feed medication (Pijpers *et al.*, 1991; Mason *et al.*, 2009), due to the fact that bivalent cations decrease the absorption and activity by chelating tetracycline (Luthman and Jacobsson, 1983).

Tylosin belongs to the macrolides. These are bacteriostatic antibiotics inhibiting bacterial protein synthesis by binding on the 50S ribosomes (Weisblum, 1998). They are active against many Gram-positive and selected Gram-negative bacteria, mycoplasmas and anaerobes (Prescott *et al.*, 2000). From a pharmacokinetic point of view, they present good absorption and tissue distribution, and accumulate principally in lysosomes of phagocytes (Scorneux and Shryock, 1998).

Both chlortetracycline and tylosin have already been shown to be efficacious to treat and control respiratory disease due to *M. hyopneumoniae* under experimental conditions (Hannan *et al.*, 1982; Ueda *et al.*, 1994; Thacker *et al.*, 2006; Vicca *et al.*, 2005), but only a few field studies are reported in the literature (Kunesh, 1981; Ganter *et al.*, 1995). The present study included a large number of pigs, measured different clinical, pathological and performance parameters, and was conducted in a herd practising vaccination against *M. hyopneumoniae* and PCV-2.

Material and Methods

Study herd

The study was conducted in a single-site farrow-to-finish Belgian pig herd, operating a one-week batch production system for the sows. There were approximately 270 commercial hybrid sows (Topigs 20), and semen from Piétrain boars was used for the inseminations. They were vaccinated against PRRSV (Ingelvac PRRS MLV®, Boehringer Ingelheim; mass vaccination twice per year), Atrophic Rhinitis (Porcilis AR-T®, MSD), *Escherichia coli* (Porcilis coli®, MSD), Porcine Parvovirus and *Erysipelothrix rhusiopathiae*, both combined according to the manufacturer's instructions (Parvoruvax®, Merial). The breeding gilts were purchased from a breeding herd, transferred to the quarantine facilities of the farm and kept there during six weeks. During this quarantine period, all gilts were vaccinated against PRRS(V) (Ingelvac PRRS MLV®, Boehringer Ingelheim; two weeks after arrival), *M. hyopneumoniae* and PCV-2 (both combined according to the manufacturer's instructions; Comboflex®, Boehringer Ingelheim), Atrophic rhinitis (Porcilis AR-T®, MSD), *Escherichia coli* (Porcilis coli®, MSD), Porcine Parvovirus and *Erysipelothrix rhusiopathiae* (Parvoruvax®, Merial).

The piglets received an iron injection (Ferraject®, Eurovet) during the first week of life. Approximately one third (31 per cent) of the males included in this study were surgically castrated, the remainder of the males were vaccinated against boar taint (Improvac®, Pfizer) when they were 10 and 18 weeks old. At three weeks of age, the piglets were weaned and vaccinated with a one-shot commercial vaccine against *M. hyopneumoniae* (Mycoflex®, Boehringer Ingelheim) and PCV-2 (Circoflex®, Boehringer Ingelheim).

At weaning, the piglets were moved to a nursery unit until 10 weeks of age, they were separated by sex and partly re-grouped according to weight. The nursery unit consisted of three compartments with four pens each and one compartment with two pens (partial slatted floor, channel ventilation, central heating with delta tubes on the walls and in the channels, 40 pigs/pen). At 10 weeks of age, they were transferred to a fattening unit and kept separated by treatment group and sex until slaughter weight (approximately six months of age). The fattening unit consisted of four compartments. Three of them had similar housing characteristics (fully slatted floor, mechanical ceiling ventilation, eight pens, 15 pigs/pen). The other one was slightly different (partially slatted floor, mechanical door ventilation, 12 pens, 15 pigs/pen). As the pen size was different between the nursery and fattening unit, the

pigs were re-grouped at 10 weeks but mixing was minimized as much as possible. During the entire experiment, water and commercial feed (meal) were supplied *ad libitum* to the pigs.

Description of clinical problems in the herd and serological data before the study

Chronic respiratory disease was commonly observed in this herd from 14 weeks of age onwards. Approximately one month before the onset of the trial, serological and pathological evaluations were carried out to establish the major pathogen(s) involved in the respiratory problems. Blood samples were taken from 15 randomly selected pigs per age group (8, 12, 16 and 20 weeks of age) to detect possible antibodies against *M. hyopneumoniae* (DAKO Mh ELISA, Dako Citomation, Denmark), PRRSV (HerdCheck PRRS ELISA, IDEXX, Idexx Laboratories, Westbrook, USA), PCV-2 (IgG and IgM, Ingezim PCV2 ELISA, Ingenasa, Madrid, Spain), Swine Influenza virus (SIV) (H1N1, H1N2, H3N2, standard haemagglutination-inhibition test) and *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) (App Serotypes 1, 9 or 11 and Serotype 2; *Actinobacillus pleuropneumoniae* 1, 9, 11 Antibody Test Kit ELISA and *Actinobacillus pleuropneumoniae* 2 Antibody Test Kit ELISA, Biovet Inc. CA). At 8 and 12 weeks of age, all pigs were serologically negative for *M. hyopneumoniae*, whereas at 16 and 20 weeks of age, all pigs had antibodies against *M. hyopneumoniae*. All pigs were seropositive for PRRSV. For PCV-2, none of the animals were positive for IgM antibodies before 12 weeks of age. At this time point, most of the animals became positive. The number of positive animals for anti-PCV-2 IgM antibodies decreased in the following weeks. The presence of PCV-2 IgG was only detected from week 12 onwards and at week 16, most of the tested animals were positive. The first seropositive pigs against SIV (H3N2) and *A. pleuropneumoniae* serotypes 1, 9 or 11 were found at 12 weeks of age. No antibodies against other SIV subtypes were found. Most of the pigs tested at 20 weeks were seropositive against *A. pleuropneumoniae* serotype 2, but not earlier. Slaughterhouse examination on 100 fattening pigs showed that 53 per cent of them had *Mycoplasma*-like lung lesions.

Study population and experimental design

In total, 533 fattening pigs were included. At the start of the fattening period (10 weeks of age), they were randomly assigned at pen level to one of the three treatment groups. Four weeks later (14 weeks of age), at the onset of disease, in-feed medication was administered (D1 or beginning of treatment). The three treatment groups were: CTC1 (500 ppm chlortetracycline chlorhydrate in-feed medication, during two consecutive weeks; Aurofac

100 mg/g Granular®, 100 mg/g, Pfizer Animal Health; n=180), CTC2 (500 ppm chlortetracycline in feed, one week medication, one week no medication and again one week medication; n=180) and T (100 ppm tylosin phosphate in-feed medication, during three weeks; Tylan 250 Vet Premix®, 250 mg/g, Eli Lilly and Company Limited; n=173). The treatment durations complied with the label directions of both products (chlortetracycline: at least one week; tylosin: three weeks) to treat and control porcine respiratory disease caused by pathogens susceptible to these products. Animals housed within the same pen were included into the same treatment group. All other factors that could possibly influence the outcome parameters, *e.g.* environment and management factors, stocking densities, sex distribution, castration versus vaccination against boar taint, feed composition (apart from the medication) were the same for the three groups. The investigator responsible of the trial was blinded for the allocation of the treatment groups. Concomitant antimicrobial treatment of study pigs (via parenteral, drinking water or feed) was only allowed for antimicrobials ineffective against *M. hyopneumoniae* such as β -lactam antibiotics (penicillins and cephalosporins). A pooled medicated feed sample was taken at the beginning of the trial from each treatment group to determine the concentration of the two antimicrobials (one sample for CTC1 and CTC2, one sample for T). The measured concentrations were very close to the recommended dose: CTC 508 mg chlortetracycline/kg, T 108 mg tylosin phosphate/kg.

The study was approved by the ethical committee for animal experiments of the faculty of veterinary medicine, Ghent University (EC2010/156).

Parameters of comparison

Performance parameters

To calculate average daily weight gain (ADG; g/pig/day) and feed conversion ratio (FCR), the individual bodyweight (BW) of all pigs was measured at three different time points: at 14 weeks (D0; onset of the clinical signs just before treatment), 17 weeks (D24; shortly after treatment) and 26 weeks (D84; end of fattening period). ADG was calculated during the treatment period (BW at D24 – BW at D0/ 25 days), the post-treatment period (BW at D84 – BW at D24/ 60 days) and for the overall period (BW at D84 – BW at D0/ 85 days). The feed intake was recorded at pen level during the treatment period. The FCR was calculated by dividing the feed intake per pen by the difference between final and starting BW. The number of dead pigs was recorded, as well as the weight and the age of each dead pig. Data from dead pigs were included in the analysis in order to calculate the mortality rate.

To establish the possible cause of death, post-mortem examination of a representative number of dead pigs (9 pigs out of 12) and additional laboratory examinations (bacteriological culture and PCR testing) were done. When *Haemophilus parasuis* was suspected to be cause of death and was not isolated by bacteriological culture, PCR testing was performed on lung samples, in order to detect the presence of DNA from this bacteria (Oliveira and others 2001). DNA was extracted using a DNA kit manufactured by Qiagen (Qiamap DNA mini kit, The Netherlands).

Days of additional treatment for each pig

The total number of days that a pig was treated individually against respiratory disease was recorded. Since concomitant antimicrobial treatment was allowed only with antibiotics ineffective against *M. hyopneumoniae*, pigs with clinical respiratory symptoms were treated parenterally either with ceftiofur (Excenel RTU®, Pfizer Animal Health) or amoxicillin (Duphamox Long Acting®, Pfizer Animal Health). Concomitant antimicrobial treatment via drinking water or feed was allowed, however it was not required.

Lung lesions

The prevalence of *Mycoplasma*-like pneumonia lesions, fissures and pleuritis was recorded at slaughter from 488 pigs. *Mycoplasma*-like pneumonia lesions (active lesions) were defined as red to purplish consolidated areas on the cranial-ventral parts of the apical, cardiac, accessory and diaphragmatic lobes. Fissures (recovering lesions) were defined as red to purplish interlobular scar retractions with the same location as *Mycoplasma*-like lesions. Pleuritis was defined as fibrotic adherence between the visceral and pleural membranes of the pleural sac. The severity of *Mycoplasma*-like pneumonia lesions (lung lesion score) was assessed for each individual pig. The lung lesion score was based on the total percentage of affected lung tissue, with the different lung lobes representing the following percentage of the total lung surface area: apical (10 per cent), cardiac (7 per cent), accessory (6 per cent) and diaphragmatic (30 per cent) (Morrison *et al.*, 1985). An average score was calculated for each treatment group based on the lung lesion score of the individual animals.

Respiratory disease score (RDS)

The severity of respiratory symptoms was assessed every two days during the first week of the treatment and twice per week during the remainder of the trial until slaughter age. To this end, the pigs were moved up and the number of pigs coughing per pen during 10 minutes was counted in all pens. A RDS was calculated by dividing the number of pigs per pen that

coughed during 10 min, by the total number of pigs in that pen, multiplied by 100 (Mateusen *et al.*, 2002). The RDS was summarised for three periods: pre-treatment (D-7/D0), treatment (D1/D22) and post-treatment (D22/D84) period.

Isolation of *M. hyopneumoniae* and MIC testing

In order to cultivate and isolate *M. hyopneumoniae*, affected lungs showing *Mycoplasma*-like lesions were collected at slaughter and transported to the laboratory, where they were processed according to previous reports (Friis, 1975). Isolates were identified as *M. hyopneumoniae* using a species specific PCR (Vranckx *et al.*, 2011). Minimum inhibitory concentration (MIC) testing of tylosin phosphate, chlortetracycline and oxytetracycline against *M. hyopneumoniae* was performed as described by Vicca *et al.* (2004). MIC was defined as the lowest concentration of the antimicrobial that completely inhibited visible growth. The *M. hyopneumoniae* strain ATCC 25634 (J-strain) was used as control strain. For the susceptibility testing, tylosin phosphate, chlortetracycline and oxytetracycline were tested for concentrations ranging from 0.015 µg/ml to 16 µg/ml.

Presence of *M. hyopneumoniae* in broncho-alveolar lavage (BAL) fluid and bacteriology

The BAL fluid was collected from 30 randomly selected pigs in each group just prior to the beginning of the treatment (14 weeks; D0) and after treatment (17 weeks; D25). Therefore, the animals were anaesthetised by administering intramuscularly 0.22 ml/kg of a mixture of xylazine (Xyl-M 2 %®, VMD) and zolazepam + tiletamine (Zoletil 100®, Virbac). The recovered BAL fluid was divided into three aliquots.

The first aliquot was used for detection of *M. hyopneumoniae*-organisms. DNA was extracted using a DNA easy Kit manufactured by Qiagen (Blood and Tissue kit, Belgium) and a quantitative PCR (qPCR) test was performed on the extract (Marois *et al.*, 2010). The qPCR analysis was carried out by CFX96 real-time PCR detection system (Bio-Rad), using a ten-fold dilution series of *M. hyopneumoniae* DNA in order to transform the threshold values to the number of organisms, considering values below the last dilution as negative. The second aliquot of the BAL fluid was used for bacteriological culture to detect the presence of main respiratory pathogens. It was inoculated on Columbia agar and Columbia CNA agar plates, both supplemented with 5 per cent sheep blood (Oxoid, Hampshire, UK). The Columbia agar plates were streaked with a *Staphylococcus pseudintermedius* strain to support growth of NAD-dependent bacterial species, such as *Actinobacillus pleuropneumoniae* biotype 1 and *Haemophilus parasuis*. Plates were incubated overnight in a 5 per cent CO₂-enriched

environment at 35 °C and identification of isolated bacteria was performed (Quinn *et al.*, 1994). A disk diffusion sensitivity testing was performed on isolated pathogens following the standards of Clinical and Laboratory Standards Institute (CLSI, 2008). Chlortetracycline disks were obtained from AlPharma-Pfizer and tylosin phosphate disks from Rosco (Neo-sensitabs, Denmark). Reading of the inhibition zones (in mm) was performed as recommended by the manufacturers based on clinical breakpoints, namely, diameter zones ≥ 23 mm (susceptible) and < 19 mm (resistant) for chlortetracycline, whereas diameter zones ≥ 26 mm (susceptible) and < 22 mm (resistant) for tylosin phosphate. The third aliquot was frozen (-80 °C) and kept as reserve.

Serology

Blood samples were taken from 45 randomly selected pigs per group (the same 30 pigs from which BAL fluid was collected plus 15 other pigs) at two time-points, namely before treatment (D0) and 35 days later. The sera were analysed to detect antibodies against *M. hyopneumoniae*, PRRSV, PCV-2 (IgG and IgM), SIV (H1N1, H1N2, H3N2) and *A. pleuropneumoniae* (serotypes 1, 9 or 11; serotype 2) as described previously in the clinical outbreak section.

Statistical analysis

The number of 144 animals in each treatment group permitted to assess a difference of 20 g (sd=60) in ADG and 3 points (sd=9) in lung lesion score with 95 per cent certainty and 80 per cent statistical power (Win Episcope 2.0, CLIVE, Edinburgh, UK). The ADG and lung lesion score were considered as major parameters.

The ADG, lung lesion score and qPCR were measured at the individual level, FCR and RDS at pen level. Analysis of variance (ANOVA) models were used to analyse the continuous variables ADG, FCR, qPCR (treatment effect) and DAT. In these models, treatment, compartment and sex were included as fixed factor and pen as random variable. In case of significant differences, pairwise comparisons between the different treatment groups were made using Scheffé's test. Data that did not fulfil the criteria of normality and homogeneity of variances (ADG) were analysed using non-parametric Kruskal-Wallis ANOVA. Lung lesion scores were compared between groups using a non-parametric Wilcoxon test. RDS and qPCR (time and group-effect) data were analysed using repeated measures ANOVA. Mortality rate, the percentage of pigs showing *Mycoplasma*-like lesions, fissures and pleuritis and the percentage of pigs showing *M. hyopneumoniae* antibodies and

that were PCR positive were analysed using logistic regression analysis, with treatment group and pen as fixed factors. Differences were considered as statistically significant when P-values were lower than 0.05 (two-sided test). Statistical analyses were performed using the software package SPSS version 18.0 (SPSS Institute Inc., Illinois, USA). Results are presented as mean $\pm$ sd unless stated otherwise.

Results

Performance parameters

The results of ADG, FCR and mortality are presented in Table 1. The overall ADG (g/pig/day) for CTC1, CTC2 and T groups were 659 ± 147 , 656 ± 145 and 638 ± 255 , respectively. The overall FCR for CTC1, CTC2 and T groups were 2.59 ± 0.17 , 2.32 ± 0.17 and 2.57 ± 0.22 , respectively. There were no significant differences between groups in ADG ($P=0.567$) and FCR ($P=0.511$).

Mortality rate was 1.1, 1.1 and 4.6 per cent for the CTC1, CTC2 and T groups, respectively ($P=0.076$). In total, 12 pigs died during the trial (2/12 CTC, 2/12 CTC2 and 8/12 T) and nine out of 12 were necropsied. Only pigs that died during the clinical outbreak and the following two months ($n=9$) were necropsied. The three pigs (one in each group) that died during the last month of the fattening period were not further investigated, as the cause of death was not considered to be related to the respiratory disease outbreak. A peak in mortality was registered during treatment period (first two weeks), affecting five pigs from group T (5/12). All of them had suffered from clinical respiratory disease; pathological findings showed pneumonia (5/5), fibrinous pleurisy (3/5) and meningitis (4/5), and *M. hyopneumoniae* was detected by PCR in the lungs (4/5). *Streptococcus suis* (1/5), *Pasteurella multocida* (1/5) and *Bordetella bronchiseptica* (2/5) were isolated from the lungs. *P. multocida* and *B. bronchiseptica* were isolated from the pig in which *M. hyopneumoniae* DNA was not detected. This pig showed pneumonia but no meningitis.

The four pigs that died during the two months after the treatment were distributed as follows: CTC1 (1/4), CTC2 (1/4) and T (2/4). All of them had suffered from clinical respiratory disease. Pathological findings showed pneumonia (4/4) and meningitis (2/4), and *M. hyopneumoniae* DNA was not detected by PCR in the lungs. *H. parasuis* was detected (PCR) in the lungs of one pig affected by fibrinous pleurisy (CTC1), as well as *A. pleuropneumoniae* biotype 1 (serotype 2) and a moderate viral load of PCV-2 (PCR; 2.7×10^6 organisms/gram) in the lungs of a wasted pig affected by purulent bronchopneumonia (CTC2). *S. suis* was isolated from the lungs and brain of two wasting pigs affected by bronchopneumonia and meningitis (T). *Trueperella pyogenes* (previously *Arcanobacterium pyogenes*) was isolated from the lungs of one wasting pig affected by bronchopneumonia and meningitis (T). All these bacteria isolated from necropsied animals were susceptible towards chlortetracycline and tylosin, based on the described clinical breakpoints.

Days of additional treatment of each pig

The average number of days of additional treatment per pig were 2.18 ± 1.74 , 1.65 ± 1.11 and 2.54 ± 2.10 for CTC1, CTC2 and T groups ($P=0.297$), respectively (Table 1). The use of ceftiofur/amoxicillin between groups was similar.

Table 1. Performance parameters (mean $\pm$ sd) (average daily weight gain ADG, feed conversion ratio FCR, mortality and days of additional treatment (DAT) in the different treatment groups: CTC1, CTC2 and T. Treatment started at D1 (when pigs were 14 weeks old).

	Period	CTC1 (n=180)	CTC2 (n=180)	Tylosin (n=173)	P-value
ADG (g/pig/day)	Treatment (D1-22)	542 $\pm$ 195	536 $\pm$ 183	517 $\pm$ 315	0.657
	Post-Treatment (D23-84)	704 $\pm$ 234	694 $\pm$ 339	726 $\pm$ 187	0.284
	Overall (D1-84)	659 $\pm$ 147	656 $\pm$ 145	638 $\pm$ 255	0.567
FCR	Treatment	2.59 $\pm$ 0.17	2.32 $\pm$ 0.17	2.57 $\pm$ 0.22	0.511
Mortality*	Overall	2/180 (1.1)	2/180 (1.1)	8/173 (4.6)	0.076
DAT	Overall	2.2 $\pm$ 1.7	1.7 $\pm$ 1.1	2.5 $\pm$ 2.1	0.297

*Mortality: number (per cent) of dead pigs

CTC1: chlortetracycline in feed (500 ppm) during two consecutive weeks; CTC2: chlortetracycline in feed (500 ppm) every other week during three weeks; Tylosin: tylosin phosphate in feed (100 ppm) during three consecutive weeks.

Lung lesions

The results of the lung lesions are shown in Table 2. The prevalence of *Mycoplasma*-like lesions was lower ($P=0.046$) and the prevalence of recovering lesions (fissures) was higher ($P=0.031$) in the CTC2 compared to the T group. The lung lesion scores, namely 15.0 ± 11.4 (CTC1), 13.4 ± 10.9 (CTC2) and 14.9 ± 12.3 (T), were not significantly different between the groups ($P=0.355$). There were no significant differences in the prevalence of pleuritis ($P=0.135$).

Respiratory disease score (RDS)

For all groups, the average RDS during the pre-treatment (12.92 ± 6.73) and treatment (12.97 ± 5.50) period was very similar, but it was significantly lower during the post-treatment period (6.88 ± 2.86). There were no significant differences between the groups (Table 3). The interaction terms treatment x compartment ($P=0.981$) and treatment x sex ($P=0.807$) were not significant.

Table 2. Percentage of slaughter pigs in the three groups with *M. hyopneumoniae*-like lesions (*Mhyo*), fissures and pleuritis, and severity of lung lesions expressed as lung lesion score (mean $\pm$ sd).

	CTC1 (n=166)	CTC2 (n=161)	Tylosin (n=161)	P-value
Percentage <i>Mhyo</i> -like lesions	20.5 ^{ab}	13.1 ^a	23.0 ^b	0.046
Percentage fissures	72.9 ^{ab}	78.1 ^a	66.5 ^b	0.031
Percentage pleuritis	21.7	25.5	18.0	0.133
Lung lesion score	15.0 ± 11.4	13.4 ± 10.9	14.9 ± 12.3	0.355

^{a,b} Values with different superscripts within a row are significantly different ($P<0.05$)

Treatment groups: CTC1: chlortetracycline in feed (500 ppm) during two consecutive weeks; CTC2: chlortetracycline in feed (500 ppm) every other week during three weeks; Tylosin: tylosin phosphate in feed (100 ppm) during three consecutive weeks.

Table 3. The average respiratory disease score (RDS) of the pigs during pre-treatment, treatment, post-treatment period in the three groups. RDS was based on the percentage of pigs coughing per pen during 10 minutes, 15 pigs were present in each pen.

	CTC1 (n=180)	CTC2 (n=180)	Tylosin (n=173)	P-value
Pre-treatment (D-7-D0)	13.14 ± 5.34 ^a	13.06 ± 7.53 ^a	12.57 ± 7.33 ^a	0.976
Treatment (D1-22)	12.94 ± 4.16 ^a	12.11 ± 6.16 ^a	13.86 ± 6.19 ^a	0.747
Post-treatment (D23-84)	6.93 ± 1.96 ^b	6.99 ± 2.32 ^b	6.73 ± 4.30 ^b	0.961

^{a,b} Values with different superscripts within a column are significantly different (P<0.05)

Treatment groups: CTC1: chlortetracycline in feed (500 ppm) during two consecutive weeks; CTC2: chlortetracycline in feed (500 ppm) every other week during three weeks; Tylosin: tylosin phosphate in feed (100 ppm) during three consecutive weeks.

Isolation of *M. hyopneumoniae* and MIC testing

The MIC of tylosin phosphate for the *M. hyopneumoniae* isolate obtained from the lungs of a slaughter pig was 0.12 µg/ml, of chlortetracycline 8 µg/ml and of oxytetracycline 0.5 µg/ml. For the J-strain, the following MIC values were obtained: tylosin phosphate 0.12 µg/ml, chlortetracycline > 8 µg/ml and oxytetracycline 1 µg/ml.

Presence of *M. hyopneumoniae* in broncho-alveolar lavage (BAL) fluid and bacteriology

The number of *M. hyopneumoniae*-organisms tested by qPCR in the BAL fluid was not significantly different between the groups (P=0.411). No time-effect was demonstrated on the load of *M. hyopneumoniae*-organisms in BAL fluid (P=0.732). Also, the interaction term between sampling time and treatment group was not significant (P=0.538) (Table 4).

S. suis was isolated from BAL fluid of four different pigs. Two out of these four isolates, belonging both to the T group and isolated from BAL fluid on sampled pigs, were resistant to tylosin phosphate, but not to chlortetracycline.

Serology

The percentage of seropositive pigs for *M. hyopneumoniae* before treatment was 60, 67 and 86 per cent in the CTC1, CTC2 and T groups, respectively. After treatment, the percentage of seropositive animals was 73, 80 and 86 per cent in the CTC1, CTC2 and T groups, respectively (Table 4). The serological results for the other respiratory pathogens are shown in Table 4.

Table 4. Number of pigs with serum antibodies against *M. hyopneumoniae*, PRRSV, PCV-2 (IgG and IgM), SIV (H1N1, H1N2 and H3N2) and *A. pleuropneumoniae* (serotype 1, 9, 11 and 2), and PCR testing for *M. hyopneumoniae* in the BAL fluid before (D0) and after treatment (D35).

	Before treatment (D0)			After treatment (D35)		
	CTC1	CTC2	Tylosin	CTC1	CTC2	Tylosin
Number of pigs with serum antibodies against ...	n=15	n=15	n=14	n=15	n=15	n=14
<i>M. hyopneumoniae</i>	9	10	12	11	12	12
PRRSV	14	14	14	15	15	14
PCV-2 Ig M / Ig G	3 / 5	7 / 8	5 / 6	3 / 13	3 / 15	5 / 14
SIV H1N1 / H1N2 / H3N2	1 / 0 / 5	0 / 0 / 1	1 / 0 / 7	2 / 0 / 9	1 / 0 / 7	2 / 0 / 8
<i>A. pleuropneumoniae</i> Serotype 1, 9 or 11	3	4	5	5	5	4
<i>A. pleuropneumoniae</i> Serotype 2	8	6	6	10	8	9
PCR testing for <i>M. hyopneumoniae</i>	n=10	n=10	n=10	n=10	n=10	n=10
Number of qPCR-positive pigs	8	7	8	9	9	10
Log copies of <i>M. hyopneumoniae</i> /ml (mean±sd)	3.49 ± 2.00	3.01 ± 1.98	3.58 ± 1.45	3.00 ± 1.21	3.38 ± 1.61	4.10 ± 1.14

There were no significant differences between the groups ($P>0.05$).

CTC1: chlortetracycline in feed (500 ppm) during two consecutive weeks; CTC2: chlortetracycline in feed (500 ppm) every other week during 3 weeks; Tylosin: tylosin phosphate in feed (100 ppm) during three consecutive weeks.

Discussion

The present study demonstrated that chlortetracycline, when administered in the feed during two alternative weeks starting at the onset of clinical respiratory disease, was able to decrease the prevalence of pneumonia lesions, and to numerically but not significantly reduce performance losses and clinical signs in a herd infected with *M. hyopneumoniae*. The treatment was compared with tylosin phosphate medication during three weeks in the feed. The tylosin treated group was considered as a positive control group, as the efficacy of this antibiotic to treat and control respiratory disease due to *M. hyopneumoniae* infections had been shown in previous studies. Kunesch (1981) reported an improvement of ADG after intramuscular administration of tylosin (4 mg/kg), and Vicca *et al.* (2005) demonstrated that pigs treated in-feed with tylosin (100 ppm during 21 days) had less pneumonia lesions and clinical signs following an experimental *M. hyopneumoniae* infection. It is likely that more pronounced effects of chlortetracycline medication would have been obtained when a negative (non-treated) control was used. A negative control group was not included because not treating clinically diseased pigs when efficacious products are available is ethically not acceptable, and also the pig producer would not have allowed to leave diseased pigs untreated (Dohoo *et al.* 2009).

The infection level for *M. hyopneumoniae* was high in the present herd, as evidenced by the high prevalence of slaughter pigs with pneumonia lesions and the fact that *M. hyopneumoniae* was detected in many necropsied pigs. The necropsy findings and the serological and bacteriological analyses also showed that other pathogens were involved in the respiratory problem. The number of pigs with serum antibodies against PCV-2 (IgG), SIV H3N2 and *A. pleuropneumoniae* increased during the treatment period, and also bacterial pathogens such as *S. suis*, *B. bronchiseptica*, *P. multocida* and *T. pyogenes* were found in the lungs of necropsied pigs. PRDC is a complex disease and it may be difficult to understand and interpret field outbreaks of respiratory disease, because of the multiple interactions of different pathogens with environmental conditions (Opriessnig *et al.*, 2011). Polymicrobial co-infections have been widely described in the literature being focused on the nature of the pathogens (virus vs bacteria), the role (primary vs opportunistic) and the mode of action (additive vs synergic) of these pathogens, and the sequencing of infection (concurrent vs simultaneous). All these factors have a crucial role on the nature of the mixed infections and, therefore, have implications on the large variability of this PRDC on field conditions. Co-infection of pigs with *M. hyopneumoniae* in conjunction with PRRSV (Thacker *et al.*, 1999),

PCV-2 (Opriessnig *et al.*, 2004) and SIV (Thacker *et al.*, 2001) showed potentiation and increased severity of the respiratory disease. However, during the respiratory outbreak of the present herd, no clinical signs and mortality associated to these pathogens (PRRSV, PCV-2, SIV) were seen, nor DNA was detected by PCR in dead animals. Only in one necropsied pig, a moderate PCV-2 viral load in the lungs was detected. Co-infection of pigs with *M. hyopneumoniae* in conjunction with bacteria, such *P. multocida* (Ciprián *et al.*, 1988) and *A. pleuropneumoniae* (Marois *et al.*, 2009) is also characterised by an increased severity of respiratory disease and enhancement of lesions, respectively. *P. multocida* was isolated from the lungs of only one dead pig, which was negative by PCR for *M. hyopneumoniae*. *A. pleuropneumoniae* was isolated in only one necropsied pig, nevertheless the prevalence of pleuritis at slaughter was lower than 22 per cent. Therefore, the presence of these bacteria could suggest an opportunistic infection. Analysis of the stable climate and the ventilation pattern could not elucidate major deficiencies in housing conditions or management practices (*e.g.* stocking density, management, deworming). Since the size of the nursery and the fattening unit was not the same, all-in/all-out management was not strictly followed, but only a limited number of pigs were mixed. This could also have contributed to respiratory disease by transmission and spread of pathogens. However, the statistical interaction terms (treatment x compartment and treatment x sex) were calculated for each parameter and no significant differences were assessed. The study illustrated that clinical problems of respiratory disease, requiring antimicrobial treatment, are still possible in herds without major deficiencies in housing and management conditions, and that practice vaccination against two major pathogens (*M. hyopneumoniae* and porcine PCV-2) involved in the PRDC.

The fact that chlortetracycline treatment performed similar or even better than a known efficacious treatment is in line with previous studies showing the efficacy of chlortetracycline. Ganter *et al.* (1995) reported that chlortetracycline-medicated feed (800 ppm, during 21 days) was able to reduce clinical signs of respiratory disease under field conditions, and Thacker *et al.* (2006) found that in-feed medication with chlortetracycline (500 ppm, during 14 days) was effective against an experimental *M. hyopneumoniae* infection. However, the medication schemes were definitively not completely efficacious. The RDS in all groups remained very similar during the treatment period as before onset of treatment, a considerable number of pigs had pneumonia lesions at slaughter, and *M. hyopneumoniae* could still be detected by PCR in the lung tissue. The latter was not unexpected and confirmed previous reports indicating that similar to vaccination, antimicrobial medication is not able to eliminate *M. hyopneumoniae* from the respiratory tract (Thacker *et al.*, 2006). It is not known why there

was no significant decrease in RDS after medication had started. It may be due to the fact that other pathogens *e.g.* viruses or bacteria not susceptible to the antimicrobials, were involved in the respiratory problem. The high mortality in the T group, nearly significantly different from the other groups, may be due to the presence of concurrent other bacterial pathogens such as *P. multocida*, *A. pleuropneumoniae*, which are less susceptible to tylosin (Barigazzi *et al.*, 1994) or *S. suis* strains with acquired resistance to tylosin. Indeed two *S. suis* strains isolated from BAL fluid showed resistance to this antimicrobial.

Based on the MIC testing performed by Vicca *et al.* (2004), the *M. hyopneumoniae* strain collected at slaughter did not show acquired resistance to tylosin and oxytetracycline. However, a high MIC value of chlortetracycline was obtained, but MIC testing of chlortetracycline is not reliable as the antimicrobial is not stable during the cultivation procedure. When chlortetracycline is incorporated in broth medium and rehydrated, it is quickly degraded: activity reduces to 1/3 after 12 h, to less than 10 per cent after 24 h and to about 2 per cent after 72 h (Pommier, 2006). Growth of *M. hyopneumoniae* on the other hand takes several days. Williams (1978) found a lower *in vitro* susceptibility of chlortetracycline against *M. hyopneumoniae* compared to oxytetracycline and doxycycline. Inamoto *et al.* (1994) also observed higher MIC values of chlortetracycline than of oxytetracycline against *M. hyopneumoniae*. Other studies also showed high MIC values (≥ 40 µg/ml) (Yamamoto and Koshimizu, 1984) or a high variation in the MIC values (1.6 – 25 µg/ml) of chlortetracycline (Etheridge *et al.*, 1979). Our finding that the *M. hyopneumoniae* isolate did not show acquired resistance to oxytetracycline strongly indicates that acquired resistance was also not present against chlortetracycline, since there exists cross-resistance between both antibiotics (CLSI, 2008).

There were only small and non-significant differences between the CTC1 and CTC2 group, but for most parameters (*e.g.* prevalence and severity of pneumonia lesions), the CTC2 group performed slightly better. An exact explanation is not available, but it may be due to the fact the pigs of the CTC2 had a better possibility to generate an active immune response during the non-medicated week in between the two medicated weeks. Walter *et al.* (2000) stated that metaphylactic pulse medication might permit a sufficient natural exposure to the pathogen and more effective stimulation of active immunity against *M. hyopneumoniae* when compared to continuous medication. Continuous medication may lead to animals that might remain immunological naïve and potentially susceptible to subsequent re-exposure to *M. hyopneumoniae* when the medication is withdrawn.

Chlortetracycline hydrochloride, when administered via the feed at the dose of 500 ppm during two alternative weeks at onset of clinical outbreak of respiratory disease, decreased the prevalence of *M. hyopneumoniae*-like lesions, and numerically reduced performance losses and clinical signs compared with tylosin phosphate at the dose of 100 ppm administered during three weeks. Vaccination and medication may significantly reduce but do not prevent infections with *M. hyopneumoniae*, implying that optimal housing and management practices remain important to keep the infection pressure at acceptable levels.

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Chapter 4. **GENERAL DISCUSSION**

General discussion

GENERAL DISCUSSION

Respiratory disease in pigs is one of the most important problems in modern pig production worldwide. It mostly results from infections with primary and opportunistic infectious agents, both viral and bacterial pathogens. Adverse environmental and management conditions may exacerbate this mixed respiratory disease resulting in the PRDC. *M. hyopneumoniae* is considered as one of the main pathogens involved in the PRDC. Infections with *M. hyopneumoniae* are present in almost all countries with an intensive swine production, and they lead to major economic losses due to the reduced growth, increased mortality and feed conversion, costs for antimicrobials and vaccination and increased time to market weight (Maes *et al.*, 2008).

The control of *M. hyopneumoniae* infections can be accomplished by optimization of management practices and housing conditions, antimicrobial treatment and vaccination. The present thesis investigated the treatment and control of *M. hyopneumoniae* infections. First, the efficacy of a new florfenicol formulation to treat pigs by means of a single intramuscular injection against experimental *M. hyopneumoniae* infection was investigated (chapter 3.1). Secondly, the efficacy of a one-shot vaccination against *M. hyopneumoniae* applied at either one or three weeks of age in a Belgian farrow-to-finish pig herd with mixed respiratory disease late in the fattening period and including infections with *M. hyopneumoniae* and viral pathogens was studied (chapter 3.2). Thirdly, the efficacy of in-feed medication with chlortetracycline and tylosin phosphate against a clinical outbreak of respiratory disease in fattening pigs that were vaccinated against *M. hyopneumoniae* was compared (chapter 3.3).

The present chapter will provide a general discussion of the findings obtained in the different studies. We will successively focus on **(1)** the major parameters used in this thesis to assess efficacy, **(2)** the different antimicrobial treatments (chapter 3.1 and 3.3), and **(3)** the different vaccination strategies (chapter 3.2 and 3.3) applied in this thesis. Finally, **(4)** general conclusions and **(5)** perspectives for further research are provided.

Assessment of efficacy under in vivo (experimental, field) conditions

This thesis includes the assessment of efficacy of antimicrobial treatment and vaccination against *M. hyopneumoniae* infections in different *in vivo* studies, conducted either under experimental or field conditions.

The importance of the measured efficacy parameters may depend whether a study is conducted under experimental or field conditions. Therefore, a table with the main advantages and disadvantages of field and experimental studies is first shown (Table 1).

Table 1. Comparison between *in vivo* studies, both laboratory and field trials.

	Laboratory experiment	Field trial
Housing conditions	controlled	real
Animals	homogeneous	heterogeneous
Management	controlled	real
Disease	model	real
Infection dose	standard for all animals	unknown
Time of infection	standard for all animals	unpredictable
Concurrent diseases	avoided	present
Variability of the outcome	low	high
External validity of the study	limited	high

Different parameters can be measured to assess efficacy of treatment and control measures against *M. hyopneumoniae* infections. The most commonly used parameters are clinical signs, lung lesions and in case of field studies also performance data.

Coughing is the main clinical sign of respiratory disease. Clinical examination of pigs affected by respiratory disease does not provide a specific aetiological diagnosis. Non-productive coughing is not a pathognomonic sign of *M. hyopneumoniae* infection, but it is commonly associated with this pathogen. The assessment of the RDS in laboratory experiments may be useful to evaluate the severity of the challenge infection, to determine the moment of the intervention (mainly treatment) and to assess the efficacy of this intervention. Regarding field experiments, it can be used in the diagnosis of enzootic pneumonia at onset of respiratory disease (Nathues *et al.*, 2012). A quantification of the severity of coughing, for instance by using a RDS, may indeed be an important indicator. Scoring the respiratory symptoms by means of a RDS may be valuable to select an appropriate antimicrobial treatment or vaccination strategy (type of product, route of administration, duration of treatment) required to treat and control respiratory disease (*e.g.* acute coughing present in a few pigs from the same pen should be treated at individual or at pen level), but also in the assessment of the clinical effectiveness of the treatment/vaccination established.

Lung lesions, such as pneumonia and adhesive pleurisy, are common findings in slaughter pigs reared under field conditions and both have been associated with respiratory disease (Fraile *et al.*, 2010; Meyns *et al.*, 2011; Fablet *et al.*, 2012b; Merialdi *et al.*, 2012). Slaughter examinations (prevalence of pneumonia and pleurisy lesions) are an excellent method to monitor the respiratory health in a herd and to evaluate the efficacy of treatments/vaccinations applied under field conditions. However, this technique has some limitations. The lack of diagnostic specificity and the subjectivity of the visual assessment are major disadvantages (Sibila *et al.*, 2009). Subclinical infections may not be detected and lesions occurring early in the fattening period may be healed by the time of slaughter (Regula *et al.*, 2000). In most experimental studies, only a limited number of animals are available due to practical and economic reasons. The current EU regulations on experimental animals also indicate to limit the number of laboratory animals as much as possible because of animal welfare reasons. However, a low number of animals can lead to not finding possible differences between groups. This disadvantage may partially be overcome by estimating the extent of lung lesions in each animal, as well as by evaluating the extent and severity of microscopic lung lesions (*e.g.* percentage of air in lung tissue, severity of peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation).

Preventing the performance losses is the main goal when treatment/vaccination against *M. hyopneumoniae* infections are established under field conditions. Although performance parameters are also commonly measured under experimental conditions, usually, no significant differences are obtained due to the limited number of animals used. Challenge infection models, are only able to provide a general tendency regarding the differences between treated and non-treated/vaccinated and non-vaccinated animals. When looking for biological, relevant and statistically significant differences, a much larger number of animals reared under commercial conditions should be included.

Serological examinations are mainly used in both experimental and field trials as a descriptive parameter to better understand the dynamics of the infection in the studied population. However, nowadays this parameter is not a good indicator to assess efficacy of current commercial vaccines against *M. hyopneumoniae*, since vaccination does not always induce seroconversion and the presence of antibodies induced after vaccination is not correlated with protection from colonization and disease (Djordjevic *et al.*, 1997). Nevertheless, serological examinations can also provide information regarding other pathogens circulating within the population and causing disease.

In vivo trials, especially when conducted under field conditions, may be largely influenced by environmental conditions. To provide standard climatic environment and management conditions to the animals will minimize the effect of this variability on the outcome of the experiments. Control systems are already available on the market to control and prevent adverse conditions. The installation of sensors capable to measure variation in temperature and ventilation, as well as feed and water intake, may be useful to understand respiratory disease course and to detect failures in antimicrobial treatment, due to deficient administration/intake, respectively. This early detection would favour an early prevention or correction of these treatment failures. Application of computerized systems may help also to reduce the variation due to subjective measurements. A smart monitoring system has been recently developed by the University of Leuven, the “Pig Cough Monitor” (Exadaktylos *et al.*, 2008). This is based on the detection of sounds produced by pigs, recognition of coughing and therefore, monitoring of frequency of this coughing. Similar applied technology based on visual detection may be also useful for recognizing macroscopic lung lesions at slaughter, and therefore for calculation of the extent of pulmonary lesions in slaughter pigs.

Antimicrobial treatment and control of *M. hyopneumoniae* infections

It is generally accepted that optimization of the housing and management conditions should be the first to be accomplished for the prevention and control of enzootic pneumonia. However, even in herds with optimal management and housing conditions, clinical outbreaks of *M. hyopneumoniae* can still occur (Vicca *et al.*, 2002). Thus, antimicrobial treatment remains an important tool to control respiratory disease caused by this bacterium.

Additionally, under field conditions, infections with *M. hyopneumoniae* are commonly accompanied by other bacterial infections (Sorensen *et al.*, 1997; Maes *et al.*, 1999; Maes *et al.*, 2000) that may increase the severity of the disease and the lesions. Antimicrobial treatment may be effective also against bacterial respiratory pathogens other than *M. hyopneumoniae*, and it can be implemented faster and in a more flexible way compared to vaccination. However, antimicrobial treatment is able to reduce the number of *M. hyopneumoniae* organism from the lung tissue, but complete elimination of this pathogen cannot be achieved (Vicca *et al.*, 2005; Thacker and Minion, 2012).

The *in vitro* activity of all three antibiotics tested in this thesis, florfenicol (chapter 2.1), chlortetracycline and tylosin (chapter 2.3), has been widely investigated. It has been shown that *M. hyopneumoniae* is intrinsically susceptible to all three antimicrobials (Prieb and Schwarz, 2003; Vicca *et al.*, 2004). However, *in vivo* activity seems to vary, likely due to the

location of *M. hyopneumoniae* at the cilia, where therapeutic levels of antimicrobials may not be easily reached (Vicca, 2005). An antimicrobial may only be effective against *M. hyopneumoniae* infection when it is able to achieve significant levels within the mucous and fluids of the respiratory tract (Thacker and Minion, 2012). Therefore, *in vivo* studies are still needed to evaluate the efficacy of antimicrobials against this pathogen.

Our first study (3.1) showed that florfenicol applied once intramuscularly at the onset of disease was effective in controlling the acute disease caused by an experimental challenge with *M. hyopneumoniae* organisms. It is well known that observations from experimental challenges must be interpreted with caution before generalization to field conditions. The amount of *M. hyopneumoniae* organisms inoculated during experimental challenge (7 mL of inoculum containing 10^7 CCU/mL) is high, but the infection takes place only once. Under field conditions, the infection dose is not known, but pigs are exposed for several days and weeks to the infection. Additionally, under experimental conditions, the inoculum is directly placed in the trachea, whereas in case of natural infections, the first contact surface is the nasal mucosa. Clinical and acute outbreaks of respiratory disease are characterized by the lack of appetite in affected animals, and consequently by a reduction of the feed and water intake. Therefore oral medication is less appropriate in controlling acute outbreaks, and individual (parenteral) treatment, *i.e.* intramuscular injection, would be required.

Immediately after intramuscular injection, florfenicol presents a high initial blood concentration as well as a high tissue distribution (bioavailability approximately 100 per cent) (Liu *et al.*, 2003). This rapid distribution (mean distribution half-life: 0.15 h) ensures a quick initial response, making this antimicrobial suitable for the treatment of acute outbreaks of *M. hyopneumoniae* infections. Florfenicol has a high *in vitro* antimicrobial activity against *M. hyopneumoniae* (Hannan *et al.*, 2000; Wilhelm *et al.*, 2012). Florfenicol is eliminated slowly (mean elimination half-life: 14h), enabling therapeutic plasma levels up to 53h (Liu *et al.*, 2003). This guarantees a long acting effect, which makes it particularly convenient to treat nursery and fattening pigs. Additionally, the therapeutic plasma level remains above the MIC of important other respiratory bacteria (*A. pleuropneumoniae*, *P. multocida*, *H. parasuis*) for at least 48h. This ensures a prolonged protection, which may enable the immune system to control secondary bacterial infections (Voorspoels *et al.*, 1999).

Scarce data is available in the literature regarding the clinical efficacy of florfenicol in pigs (Dowling, 2013). A recent field study in a pig herd infected with *M. hyopneumoniae* (Ciprián *et al.*, 2012) showed that in-feed medication with florfenicol during 35 days significantly improved performance. However, such a prolonged antimicrobial therapy may

select resistant bacteria. Thus, if a single injection at onset of disease is effective to treat *M. hyopneumoniae* in pig herds, this could significantly reduce the antimicrobial use in pig herds in comparison with oral treatments which are usually established for longer periods of time.

The florfenicol formulation used in chapter 3.1 differs from the reference veterinary medicinal product by the higher concentration of active substance (450 mg/mL), the single administration but also by a different therapeutic indication. Metaphylaxis is considered superior to prophylaxis in terms of a rational and minimized use of antimicrobials (Catry *et al.*, 2008). A metaphylactic medication (single injection of florfenicol) established at onset of disease and characterized by a higher concentration of active substance may guarantee low/very low risk of mutant selection bacteria (Drlica, 2003; Burch, 2012), while providing clinical efficacy. Although this metaphylactic strategy has been already demonstrated efficacious to treat bovine respiratory disease (Catry *et al.*, 2008), no reports are available regarding porcine respiratory disease.

The treatment significantly decreased the RDS in treated pigs, especially two days post-treatment. However, the clinical symptoms increased again four days later. Also, at necropsy only a numerical benefit was observed for the macroscopic and the histopathological lung lesions. Our findings suggested that a single injection with this antibiotic only partially controls *M. hyopneumoniae* infection during a period of approximately four days.

The performance losses and mortality were numerically improved in the treated group. Including more animals might result in statistically significant improvement, but the tendencies indicate that an economical improvement might be obtained when florfenicol is injected.

All infected pigs were positive by nPCR in the BAL fluid, indicating that the challenge infection was successful and that the treatment did not eliminate *M. hyopneumoniae* from the lungs. The latter confirms previous reports, stating that antimicrobial treatment does not eliminate the pathogen from the lung tissue nor heal existing lesions (Thacker and Minion, 2012). Antimicrobial agents may delay infection, but cannot avoid colonization, nor assure total elimination of *M. hyopneumoniae*.

Our third study (3.3) showed that chlortetracycline administered via the feed during two consecutive weeks at onset of clinical outbreak of respiratory disease was effective in reducing the prevalence of pneumonia, the performance losses and the severity of clinical signs in a herd infected with *M. hyopneumoniae* and that practiced vaccination against *M. hyopneumoniae*. The chronic respiratory disease observed in this problem herd was associated with a high infection level of *M. hyopneumoniae* that was evidenced by the high

prevalence of pneumonia at slaughter and the large proportion of *M. hyopneumoniae* PCR-positive pigs at necropsy. Also other respiratory pathogens were involved in the respiratory problems. Despite all the pigs were vaccinated against *M. hyopneumoniae*, sporadic acute outbreaks of respiratory disease prior to the study were observed at 14 weeks of age. Consequently, the medication can be considered as a metaphylactic treatment at group level. Clinically diseased animals, as well as subclinically infected animals were treated. From the viewpoint of prudent use of antimicrobials, strategic or preventive medication during periods at risk should be minimized and priority should be given to vaccination. However, as shown in the present herd, even in vaccinated pigs, clinical symptoms/outbreaks may occur (Mateusen *et al.*, 2002) and antimicrobial medication may be necessary.

Both chlortetracycline and tylosin medications administered in chapter 3.3 complied with the label directions of both products (chlortetracycline: between 7 to 14 days; tylosin: at least 21 days). However, two different treatments strategies were investigated for chlortetracycline medication: CTC1 (chlortetracycline in feed, two consecutive weeks) and CTC2 (two non-consecutive weeks, with a non-medicated week-interval in between). All three metaphylactic medications established at onset of disease and characterized by a high concentration of active substance may guarantee low/very low risk of mutant selection bacteria (Drlica, 2003; Burch, 2012), while providing clinical efficacy. However, this hypothesis has not yet been proven with *M. hyopneumoniae*. Additionally, both chlortetracycline medications (two weeks) shortened the duration of treatment when compared with tylosin medication (tylosin in feed, three consecutive weeks), hence reducing the risk of selection mutant bacteria. Although chlortetracycline has been proven efficacious in the control of porcine respiratory disease, no reports are available comparing the efficacy of this antimicrobial agent administered during two consecutives or alternating weeks.

Both chlortetracycline (Thacker *et al.*, 2006) and tylosin (Hannan *et al.*, 1982; Ueda *et al.*, 1994; Vicca *et al.*, 2005) have already been shown to be efficacious to treat and control respiratory disease due to *M. hyopneumoniae* under experimental conditions, but only a few field studies are reported in the literature (Kunesh 1981; Ganter *et al.*, 1995). In chapter 3.3, the efficacy of in-feed medication with chlortetracycline and tylosin phosphate against a clinical outbreak of respiratory disease in fattening pigs was compared. The study included a large number of pigs, measured different clinical, pathological and performance parameters, and was conducted in a herd practising vaccination against *M. hyopneumoniae* and PCV-2.

Chlortetracycline, administered by *ad libitum* intake of medicated feed, has been shown to quickly (after 2h) reach peak serum concentrations. It has a good diffusion in lung tissue

(Kilroy *et al.*, 1990; Del Castillo *et al.*, 1998; Li *et al.*, 2008). These two features ensure a quick and targeted initial response, making this antimicrobial suitable for the treatment of outbreaks of *M. hyopneumoniae* infections. Tetracyclines have been demonstrated to have a good *in vitro* activity against *M. hyopneumoniae* (Hannan *et al.*, 2000). Inamoto *et al.* (1994) reported acquired antimicrobial resistance to tetracyclines occurring in *M. hyopneumoniae* field strains isolated in Japan. Generally speaking, results of *in vitro* susceptibility testing of *M. hyopneumoniae* for chlortetracycline should be interpreted with caution, since MIC testing of chlortetracycline is not reliable as the antimicrobial is not stable during the cultivation procedure, *i.e.* it quickly degrades (activity reduced to 2 per cent after 72h) (Pommier *et al.*, 2006) and *M. hyopneumoniae* grows slowly (more than 72h). Chlortetracycline is classified as a short-acting tetracycline due to its short half-life of elimination (4h) (Del Castillo *et al.*, 1998). This quick elimination justifies the use of in-feed medication in order to maintain therapeutic levels in diseased animals for a sufficiently long time. Additionally, chlortetracycline has also been shown to be effective and is recommended for the treatment of other respiratory bacteria, such as *P. multocida* and *H. parasuis* (Burch, 2012).

Tylosin phosphate, administered via the feed, has been shown to present a good absorption and tissue distribution. Tylosin can be detected in plasma already 15 min after oral administration and the peak serum concentration are reached fast (after 4h) (summary of products characteristics). It presents a good lipid solubility, which favors its wide distribution among all tissues except for the brain (Guiguère, 2013). Tylosin is concentrated in the lungs and accumulated principally in lysozymes of macrophages (Scorneux and Shryock 1998). These characteristics ensure a quick and targeted initial response against respiratory bacterial infections. Tylosin has a good *in vitro* activity against *M. hyopneumoniae* (Hannan *et al.*, 2000). However, Vicca *et al.* (2004) reported acquired antimicrobial resistance in one out of 21 *M. hyopneumoniae* field strains isolated in Belgium. The elimination half-life of tylosin in pigs is similar to chlortetracycline (approximately 4h) (Giguère, 2013). This quick elimination justifies the use of in-feed medication in order to maintain therapeutic levels in diseased animals for a sufficiently long time. Additionally, tylosin seems to be effective for the treatment of other respiratory bacteria (*A. pleuropneumoniae*, *P. multocida*, *H. parasuis*), although MICs are intrinsically high for these *Pasteurellaceae* (Burch, 2012).

The bioavailability of antimicrobials after oral administration can be influenced by several factors, such as the pH of the stomach and gastric emptying. Tetracyclines present a low systemic bioavailability after oral administration, especially in non-fasting pigs (Del Castillo *et al.*, 2013). The presence of mycotoxins may also alter the bioavailability of

antimicrobials administered *per os* (Goossens *et al.*, 2012b). Recent *in vitro* studies showed that mycotoxins can damage intestinal epithelial cells resulting in increased passage of drugs (Goossens *et al.*, 2012a). This was confirmed under *in vivo* conditions, where administration of feed contaminated with deoxynivalenol and T-2 toxin resulted in significant increased plasma concentrations of doxycycline (Goossens *et al.*, 2012b) and chlortetracycline (Goossens *et al.*, 2013), respectively.

The tylosin treated group was considered as a positive control group, as the efficacy of this antibiotic to treat and control respiratory disease due to *M. hyopneumoniae* infections had been shown in previous studies (Kunesh, 1981; Vicca *et al.*, 2005). A negative control group was not included because not treating clinically diseased pigs when efficacious products are available is ethically not acceptable, and also the pig producer would not have allowed to leave diseased pigs untreated (Dohoo *et al.*, 2009). It is likely that more pronounced effects of chlortetracycline medication would have been obtained if a negative (non-treated) control had been used.

The efficacy of chlortetracycline treatment was in line with previous field (Ganter *et al.*, 1995) and experimental (Thacker *et al.*, 2006) studies. However, it is clear that the medication schemes were definitively not completely efficacious. The RDS in all groups remained very similar during the treatment period and a considerable number of pigs had pneumonia lesions at slaughter. Despite antimicrobial medication, *M. hyopneumoniae* could still be detected by PCR in the lung tissue. It is not known why there was no significant decrease in RDS after medication had started. Other pathogens, *e.g.* viruses or bacteria not susceptible to the antimicrobials may have been involved in the respiratory problem. The high mortality in the treated pigs may be due to the presence of concurrent other bacterial pathogens such as *P. multocida*, *A. pleuropneumoniae*, which present intrinsically high MICs for tylosin (Barigazzi *et al.*, 1994) or *S. suis* strains with acquired resistance to this antimicrobial. Indeed two *S. suis* strains isolated from BAL fluid showed resistance to tylosin.

Based on the MIC testing procedure used by Vicca *et al.* (2004), the *M. hyopneumoniae* strain collected at slaughter did not show acquired resistance to tylosin and oxytetracycline. Although a high MIC value of chlortetracycline was obtained, our finding that the *M. hyopneumoniae* isolate did not show acquired resistance to oxytetracycline strongly indicates that acquired resistance was also not present against chlortetracycline, since cross-resistance exists between both antibiotics (CLSI, 2008).

There were only small and non-significant differences between the CTC1 and CTC2 groups, but for most parameters (*e.g.* prevalence and severity of pneumonia lesions), the CTC2 group performed slightly better. An exact explanation is not available, but it may be due to the fact the pigs of the CTC2 group had a better possibility to generate an active immune response during the non-medicated week in between the two medicated weeks. Walter *et al.*, (2000) stated that metaphylactic pulse medication might permit a sufficient natural exposure to the pathogen and more effective stimulation of active immunity against *M. hyopneumoniae* when compared to continuous medication. These authors suggested that continuous medication might lead to animals that remain potentially susceptible to subsequent re-exposure to *M. hyopneumoniae* when the medication is withdrawn.

Analysis of the stable climate and the ventilation pattern could not elucidate major deficiencies in housing conditions or management practices (*e.g.* stocking density, management, deworming). This illustrated that clinical problems of respiratory disease, requiring antimicrobial treatment, are still possible in herds without major deficiencies in housing and management conditions, even if vaccination is practiced against two major pathogens (*M. hyopneumoniae* and porcine PCV-2) involved in the PRDC.

A general observation from both chapters 3.1 and 3.3, is that treatment and control of outbreaks of respiratory disease represents a very complex decision-making process, including lots of variables. When there is an acute clinical outbreak of respiratory disease, injectable treatment is necessary in those animals in which water or feed intake is reduced and therefore, are not capable to consume antimicrobials orally. This parenteral administration ensures a fast response against acute outbreaks where urgency of treatment is required. When long-acting formulations are used, the labor of repeated injections is reduced. On the other hand, oral medication is especially suited for endemic or chronic diseases, in which the appetite of the affected animals is not reduced. The presence of subclinically infected animals may pose a threat to the herd health. The administration at group level enables the treatment of the clinically and subclinically infected animals.

Vaccination against *M. hyopneumoniae* infections

Commercial vaccines are used worldwide to control *M. hyopneumoniae* infections, and in many countries vaccination is applied in more than 70 per cent of the pig herds (Maes *et al.*, 2008). Major advantages of vaccination include a reduction of performance losses, severity and extent of clinical signs and lung lesions, mortality, time to reach slaughter weight, and treatments costs (Maes *et al.*, 2008).

However, the protection conferred by commercial vaccines against *M. hyopneumoniae* is often incomplete. Several studies demonstrated that the current vaccines are not able to prevent colonization (Thacker *et al.*, 1998), to significantly reduce transmission under experimental conditions (Meyns *et al.*, 2006), and to prevent infection under field conditions (Villarreal *et al.*, 2011). Therefore, these studies suggested that vaccination alone is not able to eliminate *M. hyopneumoniae* from infected pig herds. Nevertheless, recent studies have demonstrated that vaccination may reduce the number of *M. hyopneumoniae* organisms in the respiratory tract of experimentally infected pigs (Meyns *et al.*, 2006; Vranckx *et al.*, 2012b), and therefore, may decrease the infection level in a herd (Sibila *et al.*, 2007). This further confirms that maximum beneficial effects of vaccination are reached several months after the initiation of vaccination (Haesebrouck *et al.*, 2004).

Different vaccination strategies can be implemented to control *M. hyopneumoniae* infections at herd level. Currently, one-shot vaccination is more often practiced than two-shot vaccination, mainly because it requires less labor, and it confers similar results as two-shot vaccination (Roof *et al.*, 2001, 2002; Alexopoulos *et al.*, 2004; Lillie *et al.*, 2004; Greiner *et al.*, 2011). Early vaccination (<4 weeks of age) of piglets is commonly practiced in Europe (Haesebrouck *et al.*, 2004).

Our second study (3.2) showed that a single vaccination against *M. hyopneumoniae* applied at either 7 or 21 days of age reduced the prevalence and extent of pneumonia lesions in a pig herd with clinical respiratory disease during the second half of the fattening period.

It is well known that vaccination is most likely effective if active immunity can be established before the exposure to the pathogen. It has been demonstrated that *M. hyopneumoniae* infections can take place in piglets already during the first weeks of life (Sibila *et al.*, 2007; Nathues *et al.*, 2010; Villarreal *et al.*, 2010, 2011; Vranckx *et al.*, 2012a; Fablet *et al.*, 2012a). Therefore, an early vaccination has the advantage of inducing immunity before pigs become infected, not only with *M. hyopneumoniae*, but also with other pathogens that can interfere with immune response (Maes *et al.*, 2008). The clinical course of the disease in this problem herd was characterized by a low infection level of *M. hyopneumoniae*. In previous batches before the start of the experiment, *M. hyopneumoniae* was evidenced in nursery piglets (6-7 weeks of age) by the clinical signs, the detection of *M. hyopneumoniae* PCR-positive pigs at 10 weeks of age and the presence of pneumonia at slaughter. Also other respiratory pathogens were involved in the clinical respiratory signs, but mainly during the fattening period. Consequently, this motivated the early vaccination of suckling piglets in order to control the respiratory disease in this herd.

However, the respiratory problems were different from those in the previous batches in this herd. They occurred later in the present batch, *M. hyopneumoniae* infections were less prevalent and mainly occurred from halfway the fattening period onwards, and there were complicating viral infections. This confirms that the infection pattern of *M. hyopneumoniae* is not constant over time within the same herd, and that important variations may occur between successive batches (Fano *et al.*, 2007; Sibila *et al.*, 2007). Other studies showed also a seasonal variation of *M. hyopneumoniae* infections (Straw *et al.*, 1986; Maes *et al.*, 2000; Segalés *et al.*, 2012). The absence of a constant infection pattern *i.e.* infection in younger or older pigs depending on the batch, is one of the major reasons why vaccination of piglets during the first weeks of life is commonly practiced worldwide. Using this strategy, piglets are immunized before infection, irrespective of whether infection takes place in the nursery, growing or fattening unit.

Several studies have demonstrated the efficacy of vaccination at seven days of age after *M. hyopneumoniae* challenge infection under experimental conditions in reducing lung lesions and/or clinical signs. Apart from inducing early protection, it is also important that early vaccinated pigs remain protected until the end of the fattening period, as in most pig herds, the highest infection levels of *M. hyopneumoniae* occur during the grow-finishing period (Sibila *et al.*, 2004). In this case, the question remains as to whether the effect of one-shot early vaccination under field conditions with mixed respiratory disease will last until the end of the fattening period.

Despite vaccination against *M. hyopneumoniae*, there was a very high percentage of pigs with pneumonia lesions at slaughter age (80 and 67-71 per cent in non-vaccinated and vaccinated pigs, respectively). This may likely be due to the involvement of not only *M. hyopneumoniae*, but also multiple viral infections. The infections mainly took place during the second half the fattening period, allowing insufficient time for the lesions to be healed at slaughter age (Blanchard *et al.*, 1992). Pneumonia lesions at slaughter are not pathognomonic for infections with *M. hyopneumoniae*. Apart from *M. hyopneumoniae*, swine influenza virus may cause similar pneumonia lesions (Thacker *et al.*, 2001; Sibila *et al.*, 2009). Clinical signs of respiratory disease were only present in the second half of the fattening period, while expected in nursery pigs. Additionally, because of the large number of efficacy parameters included in this experiment, it was decided to exclude the respiratory disease scoring from the efficacy assessment in order to reduce the complexity of the study design.

Both vaccination schedules led to a numerical reduction of growth losses (18-19 g/pig/day) during the fattening period. The fact that the improvement was not statistically

significant may be due to the high standard variations, the occurrence of respiratory problems only late in the fattening period, and the different viral infections that were involved in the outbreak. However, similar improvements in performance (21 g/pig/day) following *M. hyopneumoniae* vaccination were obtained in a meta-analysis of 28 published field trials (Jensen *et al.*, 2002).

Vaccination reduced the prevalence of nPCR-positive pigs, and therefore decreased the infection level of *M. hyopneumoniae* in this herd. However, *M. hyopneumoniae* DNA could still be detected by PCR. This further confirms that vaccination alone is not able to eliminate *M. hyopneumoniae* from infected pig herds (Meyns *et al.*, 2006; Villarreal *et al.*, 2011b).

It was also further confirmed that vaccination in the presence of maternally-derived serum antibodies is efficacious to control *M. hyopneumoniae* infections (Martelli *et al.*, 2006; Ritzmann *et al.*, 2006; Reynolds *et al.*, 2009). However, one-shot vaccination did not lead to significant increases in serum antibodies, confirming that serum antibody concentrations measured using the currently available ELISAs are not correlated with protection against *M. hyopneumoniae* (Djordjevic *et al.*, 1997; Thacker *et al.*, 1998). This implies that testing for the presence of serum antibodies is not reliable to evaluate whether pigs have been vaccinated properly in herds endemically infected with *M. hyopneumoniae*.

A general observation from both field studies (chapters 3.2 and 3.3) is that different vaccination schemes and commercial bacterins can be used to control *M. hyopneumoniae* infections. In chapter 3.3, despite vaccination at 3 weeks of age, clinical outbreaks of *M. hyopneumoniae* were observed, making antimicrobial treatment necessary. Vaccination against *M. hyopneumoniae* (Mycoflex®) was routinely done on this farm. In chapter 3.2, both vaccination schedules (Stellamune Once®) were able to reduce the prevalence of pneumonia; however, other viral pathogens were involved in this respiratory disease. Different commercial bacterins were used in both field studies. However, both of them have been widely tested and proven efficacious against *M. hyopneumoniae* infections. It is generally accepted that optimization of the housing and management conditions should be the first to accomplish for the prevention and control of enzootic pneumonia. However, in the herds from both field studies, analysis of stable climate and ventilation pattern could not elucidate major deficiencies in housing and management conditions. Improvement of housing and ventilation remains as milestone in the control of respiratory disease in intensive swine production systems, and therefore, further research on this topic can guarantee a better understanding of the control of the PRDC.

Table 2. Summary of the results of the main efficacy parameters assessed in Chapters 3.1, 3.2 and 3.3. Differences are presented in percentage between control and intervention groups.

	Chapter 3.1	Chapter 3.2		Chapter 3.3	
Housing conditions	experimental	field		field	
Intervention groups**	TG	V1	V2	CTC1	CTC2
Antimicrobial treatment	Florfenicol (IM)	NA	NA	Chlortetracycline (IF)	Chlortetracycline (IF)
Vaccination against <i>M. hyopneumoniae</i>	NA	1 weeks of age (Stellamune Once®)	3 weeks of age (Stellamune Once®)	***	***
Reduction (%) in:					
Respiratory disease score	-49%*	NA	NA	-7%	-13%
Prevalence of pneumonia	-10%	-11%*	-16%*	-11%	-43%*
Extent of pneumonia	-24%	-10%	-32%*	+0.7%	-10%
Increase (%) in average daily gain (g/pig/day)	+24.0%	+2.5%	+2.4%	+3.3%	+2.8%
Detection of <i>M. hyopneumoniae</i> (PCR)	Yes	Yes	Yes	Yes	Yes

*Differences between control and treatment/vaccination group were statistically significant ($P < 0.05$).

**Intervention groups: TG: pigs were injected with florfenicol 14 days after experimental challenge with *M. hyopneumoniae*; V1: pigs vaccinated at 7 days of age against *M. hyopneumoniae*; V2: pigs vaccinated at 21 days of age against *M. hyopneumoniae*; CTC1: pigs treated with chlortetracycline in feed (500 ppm) during two consecutive weeks; CTC2: pigs treated with chlortetracycline in feed (500 ppm) every other week during three weeks;

*** all pigs on the farm were vaccinated against *M. hyopneumoniae* at 3 weeks of age (Mycoflex®) as part of the management practices on the farm;

****IM: intramuscular; NA: not applicable; IF: in-feed;

General conclusions

From the studies included in this thesis (Table 2), it was concluded that both antimicrobial medication and vaccination against *M. hyopneumoniae* were able to reduce clinical signs and extent and prevalence of pneumonia lesions, as well as to improve performance in pigs infected with *M. hyopneumoniae*. However, *M. hyopneumoniae* was not eliminated from infected animals by these control strategies.

More specifically, it can be concluded that:

- The tested florfenicol formulation, when administered intramuscularly as a single dose of 30 mg/kg bodyweight, was effective in reducing the clinical respiratory symptoms in pigs experimentally infected with a highly virulent *M. hyopneumoniae* strain. A temporary reduction of clinical signs (4 days) was suggested after treatment. This metaphylactic medication characterized by a higher concentration of active substance may significantly reduce the antimicrobial use in pig herds.
- Vaccination against *M. hyopneumoniae* at either 7 or 21 days of age significantly reduced pneumonia lesions and numerically decreased growth losses in a herd with clinical respiratory problems during the second half of the fattening period due to combined *M. hyopneumoniae* and viral infections.
- Chlortetracycline hydrochloride, when administered via the feed at a dose of 500 ppm during two alternative weeks at onset of clinical outbreak of respiratory disease, decreased the prevalence of *M. hyopneumoniae*-like lesions, and numerically reduced performance losses and clinical signs compared with tylosin phosphate at a dose of 100 ppm administered during three weeks. This metaphylactic medication may significantly reduce the antimicrobial use in pig herds.
- Clinical outbreaks of respiratory tract disease requiring antimicrobial medication may still occur in herds without major deficiencies in housing and management conditions, despite vaccination against two major pathogens (*M. hyopneumoniae* and porcine PCV-2) involved in PRDC.
- Vaccination and antimicrobial medication may reduce but do not prevent infections with *M. hyopneumoniae*.

Further research

Future research on antimicrobials should be focused on *in vitro* and *in vivo* efficacy. The creation of a Mycoplasmotheque including different *M. hyopneumoniae* strains, followed by testing their antimicrobial susceptibility (MIC) to the main antimicrobials used in practice may lead to a better understanding of the *in vitro* efficacy. A subsequent correlation of this *in vitro* efficacy with *in vivo* models should be investigated. The association between antimicrobial susceptibility (MIC values) and quantification of the reduction of *M. hyopneumoniae* shedding (by qPCR) after antimicrobial treatment might be studied. This standardization would facilitate the registration of new products according to the EU laws, the establishment of correct guidelines of prudent use of antimicrobials against *M. hyopneumoniae*, as well as a reliable monitoring of the antimicrobial resistance against this pathogen. A better understanding of the pharmacokinetics may be achieved by the quantification of the antimicrobial concentration in plasma and at the site of infection. Since *M. hyopneumoniae* is a mucosal pathogen which mainly adheres to the cilia of the epithelial cells from the respiratory tract, the concentration of antimicrobial in BAL fluid and its correlation with therapeutic levels and MIC values might be used as indicators of the expected *in vivo* efficacy.

Early vaccination has been demonstrated to be efficacious against *M. hyopneumoniae* infections occurring late in the fattening period. So far, there are no reports investigating the effect of different early vaccination schemes in herds with infections occurring shortly after weaning. In addition, vaccination is often applied at weaning, as piglets are handled anyway at that time. However, weaning is also causing stress to the piglets. The effect of the stress due to weaning on efficacy of vaccination warrants further studies.

Apart from treatment and vaccination, the prevention or control of *M. hyopneumoniae* infections in pig herds by optimizing housing conditions and management practices remains important. Further studies are guaranteed in this field.

Further research to develop and validate technical devices that guarantee standard and controlled conditions along the experiments is required. Application of computerized systems able to recognize coughing of live pigs and to monitor the frequency of this coughing, as well as to recognize macroscopic lung lesions at slaughter and calculate the extent of pulmonary lesions may limit variability due to subjective measurements.

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SUMMARY

Summary

SUMMARY

Mycoplasma hyopneumoniae is the primary agent of enzootic pneumonia, a chronic respiratory disease in pigs resulting from mixed respiratory infections with *M. hyopneumoniae* and other bacterial pathogens. *M. hyopneumoniae* infections lead to major economic losses due to the reduced growth, increased mortality and feed conversion, costs for antimicrobials and immunoprophylaxis and increased time to market.

The control of *M. hyopneumoniae* infections can be accomplished by three different strategies, namely optimization of housing and management practices, antimicrobial treatment and vaccination. Both, antimicrobial treatment and vaccination have been shown to reduce infections with *M. hyopneumoniae*, but not to eliminate *M. hyopneumoniae* from infected pigs. Despite vaccination, clinical outbreaks of respiratory disease due to *M. hyopneumoniae* infections may still occur. Nevertheless, there are limited studies available describing the *in vivo* efficacy of antimicrobials under experimental *M. hyopneumoniae* induced infections. Similarly, scarce data is available regarding the efficacy of different treatment and vaccination strategies against *M. hyopneumoniae* in commercial herds infected with this bacterium and other pathogens involved in Porcine Respiratory Disease Complex (PRDC). These current control measures are beneficial from an economic point of view. Answers to these questions are necessary for a development of optimal treatment and control strategies for this disease.

The general aim of this thesis was to obtain better insights regarding different treatment and control strategies against *M. hyopneumoniae* infections.

In the first study (Chapter 3.1), the efficacy of a single intramuscular injection of florfenicol to treat clinical respiratory disease following experimental *M. hyopneumoniae* infection was investigated. *M. hyopneumoniae*-free piglets were allocated to three groups, namely, a treatment (TG) and positive control group (PCG), which were both inoculated endotracheally with a highly virulent isolate of *M. hyopneumoniae*, and a negative control group. At onset of clinical disease, TG received a single injection of a new florfenicol formulation (30 mg/kg). All pigs were euthanized 4 weeks post-infection. Clinical symptoms were significantly reduced in TG in comparison with PCG ($P < 0.05$). Average daily weight gain, feed conversion ratio, mortality and lung lesions were improved in TG compared to PCG, but the differences were not statistically significant. The results showed that, following an experimental *M. hyopneumoniae* infection, a single injection with the tested florfenicol

significantly decreased clinical symptoms and numerically improved severity of lung lesions and performance when compared to a non-treated control group.

In the second study (Chapter 3.2), the efficacy of early *M. hyopneumoniae* vaccination in a farrow-to-finish pig herd with respiratory disease late in the fattening period due to combined infections with *M. hyopneumoniae* and viral pathogens was investigated. Five-hundred-and-forty piglets were randomly divided into three groups of 180 piglets each: two groups were vaccinated (Stellamune Once®) at either 7 (V1) or 21 days of age (V2), and a third group was left non-vaccinated (NV). The three treatment groups were housed in different pens within the same compartment during the nursery period, and were housed in different but identical compartments during the fattening period. The efficacy was evaluated using performance and pneumonia lesions. The average daily weight gain during the fattening period was 19 (V1) and 18 g/day (V2) higher in the vaccinated groups when compared with the NV group. However, the difference was not statistically significant ($P>0.05$). The prevalence of pneumonia at slaughter was significantly lower in both vaccinated groups (V1:71.5 and V2:67.1 per cent) when compared with the NV group (80.2 per cent) ($P<0.05$). There were no significant differences between the two vaccination groups. In this herd with respiratory disease during the second half of the fattening period caused by *M. hyopneumoniae* and viral infections, prevalence of pneumonia lesions was significantly reduced and growth losses numerically (not statistically significant) decreased by both vaccination schedules.

In the third study (Chapter 3.3), the efficacy of chlortetracycline in-feed medication to treat pigs with clinical respiratory disease was investigated in a farrow-to-finish pig herd infected with *M. hyopneumoniae* and with clinical respiratory disease in growing pigs. In total, 533 pigs were included. The animals were vaccinated against *M. hyopneumoniae* and PCV-2 at weaning. At onset of clinical respiratory disease, they were randomly allocated to one of the following treatment groups: chlortetracycline 1 (CTC1) (two consecutive weeks, 500 ppm), chlortetracycline 2 (CTC2) (two non-consecutive weeks, with a non-medicated week-interval in between, 500 ppm) or tylosin (T) (three consecutive weeks, 100 ppm). Performance (average daily gain, feed conversion ratio), pneumonia lesions at slaughter and clinical parameters (respiratory disease score) were assessed. Only numeric differences in favour of the CTC2 group were obtained for the performance and the clinical parameters. The prevalence of pneumonia lesions was 20.5, 13.1 and 23.0 per cent ($P<0.05$) for the CTC1, CTC2 and T groups, respectively. The study demonstrated that chlortetracycline, when administered at onset of clinical respiratory disease via the feed at a dose of 500 ppm during

two alternative weeks, was able to decrease the prevalence of pneumonia lesions, and numerically reduce performance losses and clinical signs.

From the studies included in this thesis, it was concluded that both antimicrobial medication and vaccination against *M. hyopneumoniae* were able to reduce clinical signs and extent and prevalence of pneumonia lesions, as well as to improve performance in pigs infected with *M. hyopneumoniae*. Although *M. hyopneumoniae* was not eliminated from infected animals by these control strategies, metaphylactic medication and vaccination may contribute to the reduction of antimicrobial use in pig herds.

Future research on antimicrobials could focus on *in vitro* and *in vivo* efficacy, *e.g.* to better understand the association between antimicrobial susceptibility (MIC values) and quantification of the reduction of *M. hyopneumoniae* shedding (by qPCR) after antimicrobial treatment. In addition, the quantification of antimicrobial concentration in plasma and at the site of infection and its correlation with MIC values might be used as indicators of the expected *in vivo* efficacy. Interesting further research on vaccination against *M. hyopneumoniae* could address the effect of different early vaccination schemes in herds with infections occurring shortly after weaning. The effect of stress at vaccination, for example at weaning, on efficacy of vaccination warrants further studies. Development and validation of technical devices that guarantee standard and controlled conditions along the experiments may be required. Future studies might focus on the application of computerized systems to recognize coughing of live pigs as well as on macroscopic lung lesions at slaughter in order to reduce variability of subjective measurements.

SAMENVATTING

Samenvatting

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Mycoplasma hyopneumoniae is het causaal agens van een chronische respiratoire ziekte bij varkens genaamd enzoötische pneumonie. Deze ziekte ontstaat door menginfecties van *M. hyopneumoniae* en andere bacteriële pathogenen. Infectie met *M. hyopneumoniae* leidt tot grote economische verliezen, verminderde groei, een hoger sterftcijfer, hogere voederconversie, meer kosten aan profylactische en antimicrobiële middelen en de varkens hebben meer tijd nodig om slachtgewicht te bereiken.

Drie strategieën om *M. hyopneumoniae*-infecties onder controle te houden worden aangeraden: optimaliseren van huisvesting en management, antimicrobiële medicatie en vaccinatie. Deze twee laatste strategieën zijn in staat om infecties met *M. hyopneumoniae* te reduceren, maar niet te elimineren. Niettegenstaande vaccinatie wordt uitgevoerd, kunnen er nog steeds infecties met *M. hyopneumoniae* voorkomen. Er is slechts weinig onderzoek beschikbaar dat de werkzaamheid van antimicrobiële middelen tegen experimenteel geïnduceerde *M. hyopneumoniae*-infecties beschrijft. Eveneens is er weinig kennis omtrent de werkzaamheid van behandelings- en vaccinatiestrategieën tegen *M. hyopneumoniae* in commerciële varkensbedrijven waar *M. hyopneumoniae* en andere pathogenen betrokken in het Porcine Respiratoir Ziekte Complex (PRDC) een rol spelen. Deze huidige controlemaatregelen zijn interessant vanuit economisch oogpunt, bijgevolg zijn antwoorden op deze vragen noodzakelijk voor de ontwikkeling van een optimale behandelings- en controlestrategie voor deze ziekte.

Het doel van deze doctoraatsthesis was om een beter inzicht te verkrijgen wat verschillende behandelings- en controlestrategieën tegen *M. hyopneumoniae* betreft.

In de eerste studie (hoofdstuk 3.1) werd de werkzaamheid van een éénmalige intramusculaire injectie met florfenicol voor de behandeling van een experimentele *M. hyopneumoniae* infectie nagegaan. Biggen, vrij van *M. hyopneumoniae*, werden aan drie groepen toegewezen: een behandelingsgroep (BH), een positieve controlegroep (PCG) en een negatieve controlegroep. De BH en PCG werden beiden endotracheaal geïnoculeerd met een hoogvirulent isolaat van *M. hyopneumoniae*. Bij de aanvang van de klinische symptomen kreeg de BH een enkelvoudige dosis van een nieuwe florfenicol-formulatie (30 mg/kg). Alle biggen werden vier weken na infectie geëuthanaseerd. De klinische symptomen waren significant lager in de BH in vergelijking met de PCG ($P < 0.05$). De dagelijkse groei, de voederconversie, het sterftcijfer en de longletsels in de BH werden positief beïnvloed in vergelijking met de PCG, maar de verschillen waren niet statistisch significant. Uit deze

resultaten kon worden besloten dat een injectie met de geteste florfenicol-formulatie na een experimentele *M. hyopneumoniae* infectie de klinische symptomen significant deed afnemen en dat een numerieke verbetering kon gezien worden in de ernst van longlesies en productie in vergelijking met de niet behandelde groep.

In de tweede studie (hoofdstuk 3.2) werd de werkzaamheid van een vroege *M. hyopneumoniae* vaccinatie onderzocht in een gesloten bedrijf met respiratoire ziekte-tekens die optraden laat in de afmestperiode te wijten aan een combinatie van *M. hyopneumoniae* en virale pathogenen. Vijfhonderd veertig biggen werden *at random* verdeeld over drie groepen van 180 biggen: twee groepen werden gevaccineerd (Stellamune Once®) op respectievelijk 7 (V1) of 21 (V2) dagen leeftijd en een derde groep werd niet gevaccineerd (NV). In de biggen-batterij werden de biggen gehuisvest in verschillende hokken binnen dezelfde stal en tijdens de afmestperiode in verschillende maar gelijkaardige compartimenten. De werkzaamheid werd beoordeeld door middel van productieparameters en pneumoniescores. De dagelijkse groei tijdens de afmestperiode was 19 (V1) en 18 g/dag (V2) hoger in de gevaccineerde groepen in vergelijking met de NV groep, hoewel het verschil niet statistisch significant was ($P > 0.05$). De prevalentie van pneumonie aan de slachtlijn was significant lager in beide gevaccineerde groepen (V1: 71.5 en V2: 67.1 per cent) wanneer vergeleken werd met de NV groep (80.2 per cent) ($P < 0.05$). Tussen de twee gevaccineerde groepen werden er geen significante verschillen gezien. In dit bedrijf met late respiratoire symptomen te wijten aan een combinatie van *M. hyopneumoniae* en virale infecties werd dus bij toepassing van beide vaccinatieschema's een statistisch significante verbetering gezien van de pneumonie prevalentie en een numeriek (niet statistisch significant) betere groei.

In de derde studie (hoofdstuk 3.3) werd de werkzaamheid nagegaan van chloortetracycline toegediend in het voeder om varkens in een gesloten bedrijf geïnfecteerd met *M. hyopneumoniae* en met klinische respiratoire ziekte te behandelen. Vijfhonderd drieëndertig varkens werden opgenomen in de studie. De varkens werden gevaccineerd tegen *M. hyopneumoniae* en PCV-2 bij het spenen. Bij aanvang van de klinische respiratoire symptomen werden de dieren *at random* verdeeld tot een van de volgende behandelingsgroepen: chloortetracycline 1 (CTC1) (twee opeenvolgende weken, 500 ppm), chloortetracycline 2 (CTC2) (twee niet-opeenvolgende weken, met een niet-gemedicineerde week als interval tussen de twee weken, 500 ppm) en tylosine (T) (drie opeenvolgende weken, 100 ppm). Productieparameters (dagelijkse groei, voeder conversie ratio), pneumonie lesies bij slacht en klinische parameters (respiratoire ziekte score) werden beoordeeld. Voor de productie- en klinische parameters werden enkel numerieke verschillen ten gunste van de

CTC2-groep verkregen. De pneumonie prevalentie was 20.5, 13.1 en 23.0 per cent ($P < 0.05$) voor respectievelijk de CTC1, CTC2 en de T-groep. Deze studie toonde aan dat wanneer chloortetracycline toegediend via het voeder in een dosis van 500 ppm tijdens twee niet-openvolgende weken bij de aanvang van de klinische respiratoire symptomen in staat was om de pneumonie prevalentie te verminderen en om de productie parameters en klinische symptomen numeriek te verbeteren.

Uit de studies die in deze doctoraatsthesis werden beschreven, kon besloten worden dat zowel antimicrobiële middelen als vaccinatie tegen *M. hyopneumoniae* in staat waren om de klinische symptomen en de uitgebreidheid en prevalentie van pneumonie-letsels te reduceren en de productieresultaten van varkens geïnfecteerd met *M. hyopneumoniae* te optimaliseren. Alhoewel deze controlematregelen niet in staat waren om *M. hyopneumoniae* uit de geïnfecteerde dieren te elimineren, kunnen metafylactische medicatie en vaccinatie wel bijdragen tot het reduceren van gebruik van antimicrobiële middelen in de varkensbedrijven.

Verder onderzoek i.v.m. antimicrobiële middelen zou zich kunnen toespitsen op de *in vitro* en *in vivo* werkzaamheid, bv. om de associatie tussen antimicrobiële gevoeligheid (MIC-waarden) en de kwantificering van de reductie van *M. hyopneumoniae* na antimicrobiële behandeling (d.m.v. qPCR) na te gaan. Bovendien kan de kwantificering van de antimicrobiële concentratie in het plasma en t.h.v. de infectiehaard gecorreleerd met de MIC-waarde aangewend worden als indicator voor de verwachte *in vivo* werkzaamheid. Verder onderzoek aangaande vaccinatie tegen *M. hyopneumoniae* zou het effect van verschillende vroege vaccinatieschema's kunnen nagaan in bedrijven waar de biggen vroeg na het spenen geïnfecteerd worden. Er is verder onderzoek nodig naar het effect van stress, bv. tijdens het spenen, op de werkzaamheid van de vaccinatie. Ontwikkeling en validatie van technische apparatuur waarbij klinische symptomen en longletsels op een objectieve en gestandaardiseerde manier gemeten worden, kunnen hun nut hebben. Verder onderzoek kan zich aldus toespitsen op het gebruik van geautomatiseerde systemen voor het registreren van hoesten bij varkens en het herkennen van macroscopische longlesies aan de slachtlijn, om zo de variabiliteit van subjectieve metingen te verminderen.

CURRICULUM VITAE

Curriculum vitae

Rubén del Pozo Sacristán was born on June 4th 1981, in Segovia (Spain). He finalized his secondary studies in Biomedical Sciences in 1999 in the College of H.H. Maristas Ntra. Señora de la Fuencisla, Segovia. He continued his academic formation at the Faculty of Veterinary Medicine of León (Spain), where he obtained his DVM diploma in 2007.

Immediately after graduation and during two years, he was working as swine practitioner in a pig farming integration, Progatecsa, in Valladolid (Spain).

He started an internship program for the European College of Porcine Health Management in October 2009 at the Unit of Porcine Health Management, Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University. From October 2010 onwards, he followed a residency program for the same college at the same department. His interest in research led him to combine this residency with different research projects that resulted in a PhD project entitled “Treatment and control of *Mycoplasma hyopneumoniae* infections”.

Rubén is first author and co-author of several articles published in international peer reviewed journals. His experimental work has been presented during different European and international congresses.

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Once, I heard that memories are made not only by places but also by the people present at a specific moment of time in these places. These five years here in Merelbeke (by extension in Belgium) were a wonderful time and all of you are responsible of this. I cannot find a better word to thank you all:

DANKUWEL!!!

At this point, I must not forget my past coming from Spain. Prof. Tejerina, José Carlos García, as guiders of my first steps as veterinarian, I cannot overlook our experiences in Centrotec and all doors that you opened to me. Many thanks also to all Spanish practitioners/colleagues that motivated me to love pigs. My gratitude to all members of PigChamp for taking care of me during that productive stage in November 2013. A special thank to Progatecsa (Javier Llamazares, Javier Rabanal, Chema Ordoñez, Juanma Martínez and Marcial Marcos). You offered me your unconditional support during my first steps as swine practitioner. Therefore, part of my success is also yours.

Thanks to H. Agustin Montero, for his example as teacher, for his love and dedication to his job. Agustín, now I realize that your role on this story was to light a flame on me, which continuously remembered me how fortunate I am to have the possibility to learn and the opportunity to share knowledge with the rest. Continúa sonriendo como tú lo hacías.

Because in this life not everything is work, a special mention is required to MADRONA. To “Los del Corral” [Nando (79), Lele, Santines, Morenín (80), Josito, Kikines, Hugo, Davicín, Julito (81), Germán, Jaime, Javi (82), Luismi y el Indio-cabrón], because of all these 25 years together; because of all vermouths together; because of everything... Nowadays is called “teamworking”, but I am sure that this is simply friendship (al final he perdido la factura de la nevera azul, así que os la perdonaré). A las chicas del Corral, que aunque no os dejemos ser de la peña, siempre os tenemos presentes (Laura, Leti, Sara, Virgi, Ana Pastor, Valle, Hermanas Conde...). Charanga Alioli, I am very pleased to be re-enrolled into “Charangueo” with all of you. Thanks for doing music funny and not mandatory. Lara, Sara y Elena, thanks for your help on that slaughter visit; sure that you will never forget it.

A huge step into my spiritual maturation was due to all years I have been living in León, mainly in the C.M.U. San Isidoro. Pneb and Miguel were my first roommates. If I was able to get a Vet diploma, it was in part because of you (y nuestra puerta al infierno, la 201). Carlitos, Paquito, Pelayo, Pablo, Nachín, Pedro, Claver, Leandro, Penkas, Potes, Colibrí, “Abuelo Manuel”, Cabrero, Puma, Cochino, Lomillos, Sagui, Ulibarri, Dani Frasco y José Luis Zidane... tenías razón, al final lo que uno recuerda de esos maravillosos años es sólo lo bueno (lo malo se borra).

Miguel, I do not have space enough to thank all your support, moments and experiences that we lived during these 15 years of friendship. First León, then Madrid, Valladolid, Gent ... and now Brazil, I am sure that this will not be a difficulty to follow quite similar philosophy of life. *“No matter where you go, there you are”*.

Last but not least, to my family. No lo recuerdo bien, pero si alguien es el culpable de mi amor por los animales, y en especial por los cerdos, esos son mis abuelos. Abuelo Lorenzo, abuela Vitorina, abuelo Mariano y abuela Olimpia, el hecho de tener animales en casa y el hecho de compartir cada año la tradicional matanza en familia ha causado un efecto imborrable en mi infancia y estoy seguro que seguirá guiando mi futuro. Todo eso os lo debo a los cuatro. Vuestro entusiasmo y dedicación por cuidar la vida familiar es admirable y es algo que siempre llevaré conmigo. Allá donde estéis, no os preocupéis, porque sigo vuestras enseñanzas. Y recordad: *“el que pa'lante no mira, atrás se queda”*. A todos mis tíos y primos. We all are lucky for belonging to a this diverse and huge family. Thank you very much for taking care of our parents while both of us we were far away.

To my sister. Marina. Eres de las pocas personas a las que miro a la cara y sé que están pensando. Ahora me doy cuenta de que todo lo que compartimos cuando éramos niños, me ha servido para entender la vida de otra forma. Siempre hay varias perspectivas para todo, la tuya, la mía y la de Papá y Mamá. Ninguna es menos buena que las demás, pero todas son válidas.

To my parents. A mis padres. Mamá, Papá, porque todo lo que soy y todo lo que no soy os lo debo a vosotros. Nunca os podré demostrar todo lo agradecido que os estoy por todo lo que me habéis dado en esta vida. Consejos, apoyo moral, sabiduría, y el conocimiento necesario para saber decidir por mi solo. Gracias por apoyarme cuando decidí estudiar Veterinaria. Espero que este día lo recordéis siempre y que estéis orgullosos de mí. Aunque sea una persona que no expresa sus sentimientos constantemente, quiero que sepáis que me acuerdo de vosotros cada día y que os llevaré en el corazón durante toda mi vida.

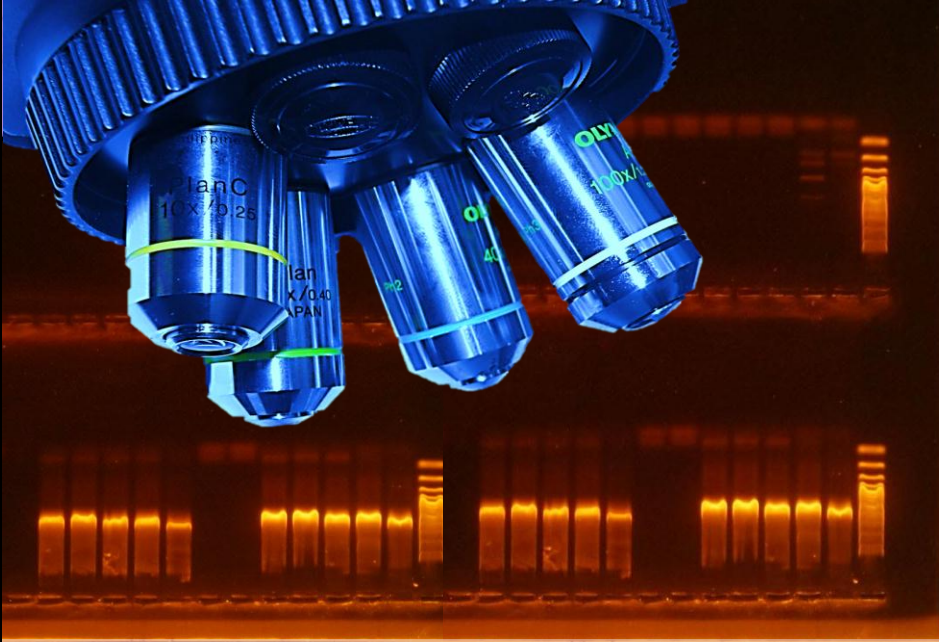
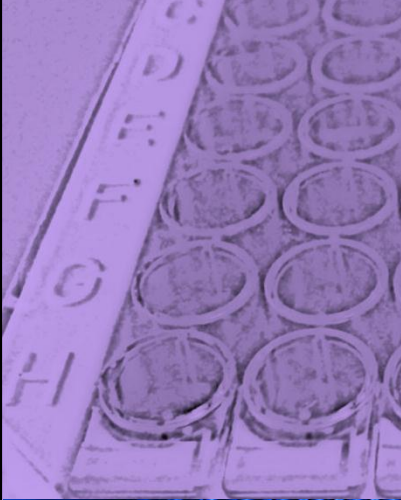
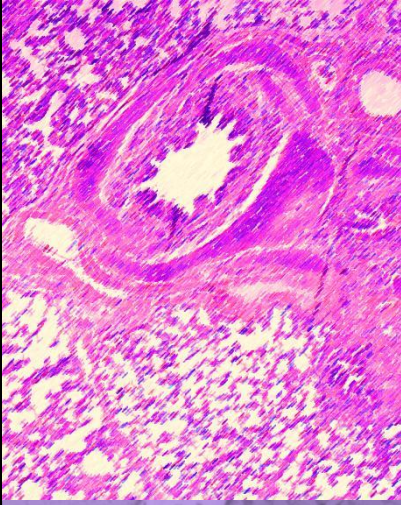
GRACIAS

Experiencia es esa cosa maravillosa que te permite reconocer un
error cuando lo cometes de nuevo

Franklin P. Jones

Experience enables you to recognize a mistake when you make it
again

Franklin P. Jones



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